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# Synthesis and AFM studies of lectin–carbohydrate self-assemblies

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ABSTRACT

Lectins are carbohydrate-binding proteins found in plants, viruses, bacteria, and animal cells having the ability to distinguish complex carbohydrate structures on cell surfaces. The ubiquity of lectin–carbohydrate interactions support the notion of a glycocode, which is related to highly specific recognition of carbohydrate display expressed on different cell surfaces of normal and diseased origin. The importance of these interactions in cell biology and medicine has been realized with the applications ranging from blood typing to clinical diagnostics. This paper reports synthesis of some symmetrical carbohydrate derivatives, their interaction with lectin Concanavalin A and ultrastructural force microscopic studies of the complex formation. Preliminary efforts have been made to construct possible models to explain the nature of interaction within these assemblies.



## 1. Introduction

Lectins serve as key carbohydrate recognition molecules, which bind reversibly to specific sugars and are implicated as versatile modifiers of important biological processes.[1] Carbohydrate recognition and binding is an important event widely used for the design and development of biological assays and drug delivery systems.[2] Carbohydrate–lectin interactions often occur with low monomeric binding affinities ($K_d = \sim 10^{-3}$ M),[3] hence multivalency is invoked to achieve physiologically relevant association constants.[4,5] High affinity lectin-binding ligands are either complex natural oligosaccharides or synthetic carbohydrate clusters.

Given the excitement in bioinspired recognition events, many groups have reported novel carbohydrate scaffolds to study lectin–carbohydrate interactions at the molecular level.[6] These studies have revealed the sites of interaction, involvement of specific amino acids in recognition and binding, and the possibility of constructing supramolecular assemblies based on these interactions.

However, limited attention is focused to probe and decipher these assemblies with the help of force microscopic tools. For example, Dufrêne and co-workers used a thiol-terminated hexasaccharide to study its interaction with a plant lectin Concanavalin A (Con A) with the help of AFM imaging and force–distance measurements.[7] They calculated characteristic elongation forces, rupture lengths, and features attributed to specific lectin–carbohydrate interactions. Another study by Sasaki and co-workers demonstrated the formation of nanoscale dendritic structures via Con A and mannose-containing glycolipid interaction by probe microscopy.[8] Recent investigations by Wang co-workers involved AFM studies of protein–carbohydrate self-assembled monolayers (SAM) interaction on gold surfaces.[9] It was inferred that carbohydrate SAMs offer convenient platform for high throughput characterization of carbohydrate–protein interactions. Other selected studies include scanning force analysis of Con A–glycoprotein interaction,[10] force microscopy-based study of micro patterns formed from Con A–carboxypeptidase Y interaction,[11] and force measurement required for the rupture of a single Con A–mannose association,[12] to name a few.

Plant lectin Con A is a well characterized mannose-specific tetrameric protein, which is believed to play a crucial role in the recognition of highly mannosylated glycans of foreign microorganisms and plant predators.[13] A 2.9-Å resolved structure of Con A–methyl-α-D-mannopyranoside complex has been reported where the carbohydrate was found anchored to the protein via hydrogen bonds and Van der Waals interactions.[14] Several others have also reported crystal structures of complexes containing mannose derivatives and Con A, suggesting subtle details of association and flexibility of lectin structure.[15]

The aim of our present study was to employ synthetic mannose-containing symmetrical molecular scaffolds to explore their interaction with Con A by the help of atomic force microscopy. It was anticipated that flexibility of Con A tetrameric structure and diverse

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spatial predisposition of synthetic mannose-containing ligands, will reveal interesting patterns, which can be interpreted on the basis of model interaction schemes.

## 2. Results and discussion

Con A binds specifically to α-D-mannopyranosyl, α-D-glucopyranosyl, and β-D-fructofuranosyl residues in polysaccharides as well as to mono and oligosaccharides bearing the same groups.[13] *N*,*N*′-Di-α-D-mannopyranosylurea (**1**) (Fig. 1) was prepared by a literature method reported previously, but herein we provide complete characterization of this compound.[16] O-Linked mannose derivatives **2** and **5** were synthesized by the condensation of fully protected mannopyranoside donors with respective alcohols according to previously reported procedures.[17,18] Conversion of the protected intermediates to the deprotected divalent and trivalent targets was obtained in excellent yield by simple treatment with sodium methoxide and methanol (Scheme 1).[17]

Target N-linked mannosyl derivatives tris[*N*,*N*-α-D-mannopyranosyl-2-aminoethyl]amine, and *N*,*N*′-di-α-D-mannopyranosyl-1,4-diaminobutane were synthesized by previously reported procedure in good yields (Scheme 2).[18] Anomeric configuration of the mannose derivatives was determined as α-anomer by 2D COSY and NOESY experiments, wherever required. These N- and O-linked saccharides were then interacted with Con A and studied with the help of atomic force microscopy.

The solutions of respective mannose conjugates with Con A were prepared and spread on mica surface to reveal distinct surface deposition patterns. Mannose conjugates were used in 20-fold excess with respect to the lectin solution as their interaction is typically weak due to shallow binding pockets in lectins that are solvent exposed.[15] Our objective was to exploit the multivalency of mannose display in lectin binding, in order to study the patterns formed due to this interaction.

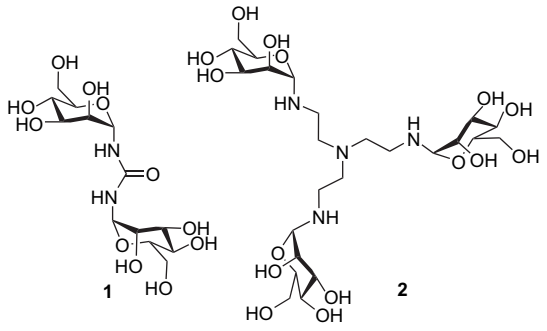
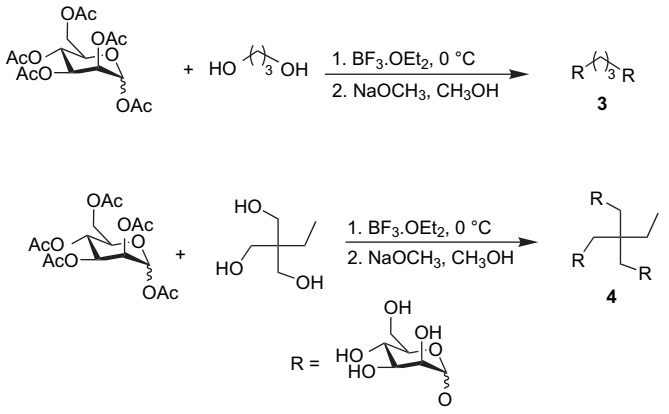
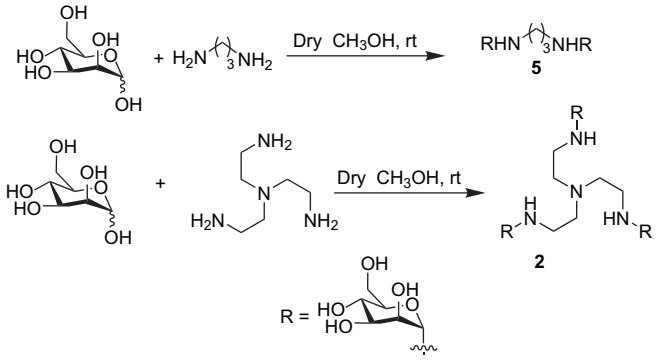


**Figure 1.** Molecular structures of *N*,*N*′-di-α-D-mannopyranosylurea (**1**) and tris[*N*,*N*-α-D-mannopyranosyl-2-aminoethyl]amine (**2**).



**Scheme 1.** Synthetic steps for preparation of O-linked mannose derivatives.



**Scheme 2.** Synthetic steps for preparation of N-linked mannose derivatives.

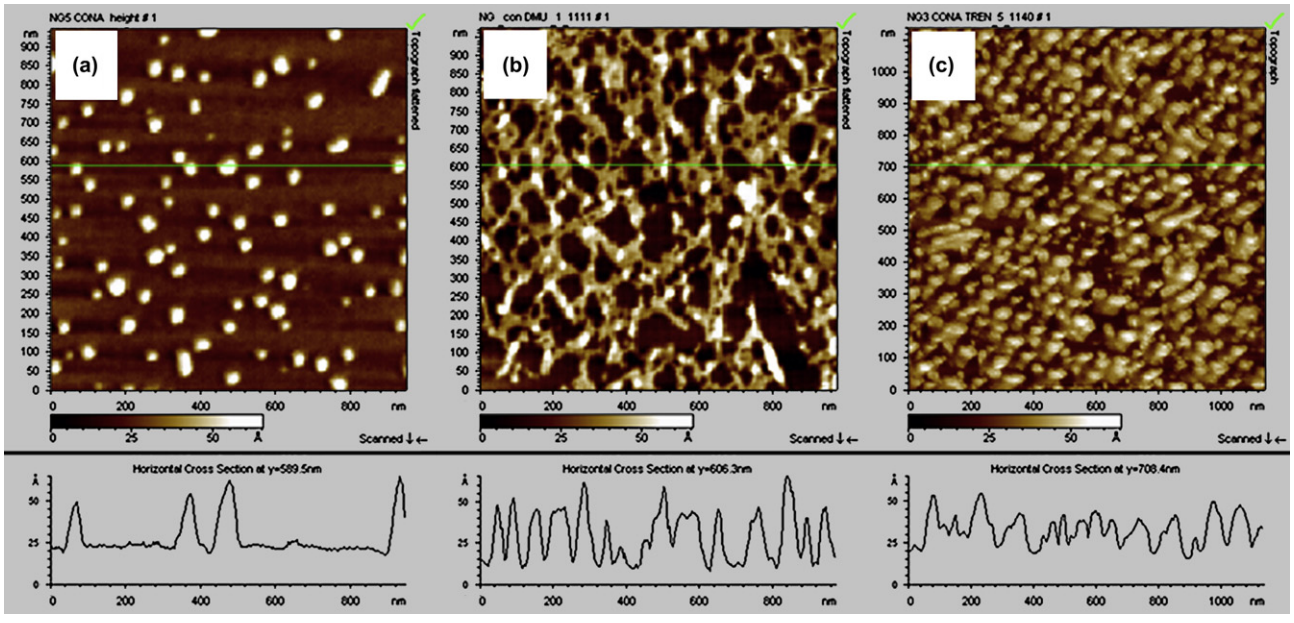



**Figure 2.** 2D AFM micrograph of (a) Con A alone, (b) Con A with **1**, and (c) Con A with **2**.

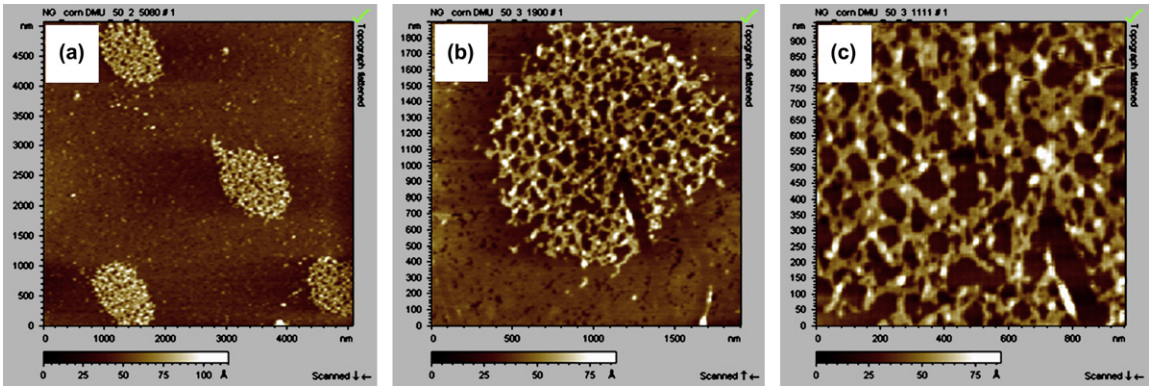



**Figure 3.** Three magnified AFM micrographs of Con A–**1** interaction.

These patterns, as determined by AFM, were particularly interesting for *N*,*N*′-di-α-D-mannopyranosylurea (**1**) and tris[*N*,*N*-α-D-mannopyranosyl-2-aminoethyl]amine (**2**). Thus, we decided to study these patterns in greater detail. From the AFM micrograph, it may be suggested that Con A upon interaction with $C_2$-symmetric **1** affords a cross-linked network while a similar interaction of Con A with $C_3$-symmetric **2** results in the formation of clusters over a wide range of surfaces studied with AFM (Fig. 2). On a closer inspection, Con A–**1** interaction revealed interesting gross morphology and it was possible to follow the surface patterns under increasing magnification range (Fig. 3).

Con A occurs as a tetramer in solution at pH 7.0 or above.[19] But, it may dissociate into dimers as a result of dilution and ionic strength at a concentration less than 5 μg/ml at pH >7.0.[20] Since the concentration of Con A used in our study was 2000 ng/ml, we assume that Con A would mainly exist as a dimer and thus, we have tried to construct possible models considering the dimeric structure of Con A (Fig. 4).

It was intriguing that the generation of uniform surface patterns were not obtained with other mannose derivatives (**3**–**5**). It is possible that the linkers might play an important role, which remains unclear at the present time (see Fig. 5). We are currently trying to study these interactions with other lectins to ascertain the consequences of specificity vis-à-vis structure of mannose conjugates.

However, we further decided to check specificity of interactions by using corresponding galactose derivatives of **1** and **2**, as it is known that Con A has minimal affinity for galactose and thus, these analogues might act as suitable controls.[21] Consequently, *N*,*N*′-di-α-D-galactopyranosylurea (**1a**) and tris[*N*,*N*-α-D-galactopyranosyl-2-aminoethyl]amine (**2a**) were synthesized to study the possibility of generating patterns with Con A incubation. As expected, the AFM micrographs of Con A–galactose conjugate interaction did not reveal any pattern formation (Fig. 6), thus leading to a conclusion that network or cluster visualized for compounds **1** and **2** with Con A could be attributed to specific interaction between mannose residues and Con A.

$Ca^{2+}$ and $Mn^{2+}$ ions are crucial for Con A–carbohydrate interactions.[22] Incubation of **1** and **2** with Con A in the absence of

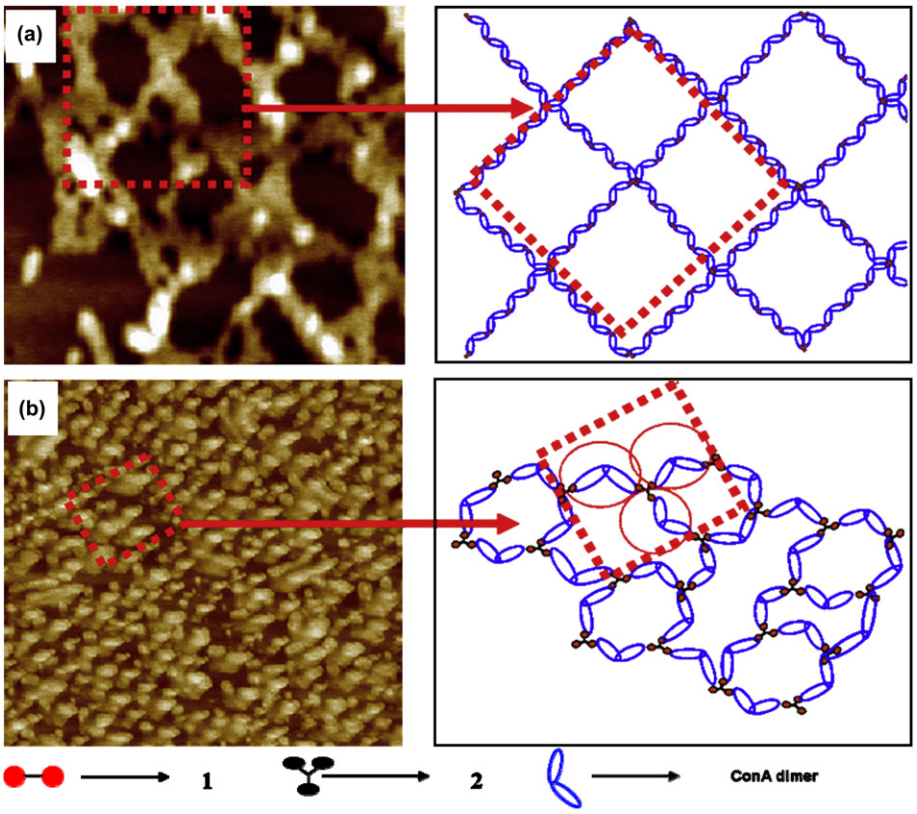



**Figure 4.** Proposed models for Con A interaction with (a) **1** and (b) **2**.

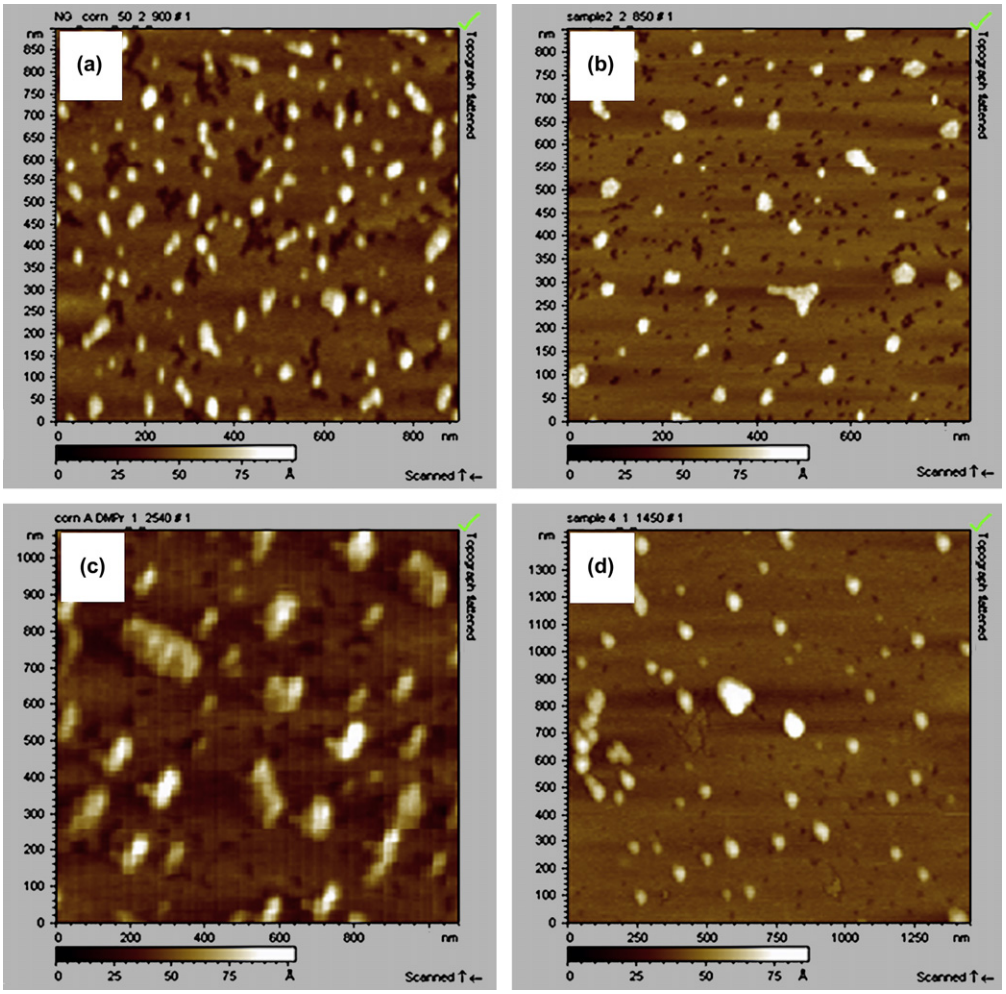



**Figure 5.** AFM micrograph of Con A with mannose derivatives (a) Con A with D(+)-mannose, (b) Con A with 1,2-bis[*O*-α-D-mannopyranosyl]propane-1,3-diol (**3**), (c) Con A with tris[*O*-α-D-mannopyranosyl]2-ethyl-2-(hydroxymethyl)-1,3-propanediol (**4**), and (d) Con A with *N*,*N*′-di-α-D-mannopyranosyl-1,4-butanediamine (**5**).

these ions failed to generate decipherable patterns as detected by the AFM analysis (Fig. 6), thus suggesting that interactions of these synthetic conjugates follow the general requirements of Con A–carbohydrate interaction.

## 3. Conclusions

*N*,*N*′-Di-α-D-mannopyranosylurea (**1**) and tris[*N*,*N*-α-D-mannopyranosyl-2-aminoethyl]amine (**2**) showed strong interactions with Con A as studied through AFM. Our results suggest that small synthetic mannose conjugates could act as potential ligands for Con A binding, in contrast to previously reported highly branched mannose dendrimers. Future studies in this direction will follow interaction of synthetic carbohydrates conjugates with lectins for the generation of complex assemblies and directed surface patterning as part of our ongoing investigations concerning self-assembly process,[23] and are expected to complement other biophysical studies of lectin-multivalent carbohydrate interactions reported in literature.[24]

## 4. Experimental

### 4.1. General

$^{1}$H and $^{13}$C NMR were recorded by JEOL-JNM LAMBDA 400 model operating at 400 and 100 MHz, respectively. The exact positions of different hydrogens of mannose were determined by 2D COSY, wherever required. Anomeric configuration and exact stereochemistry were determined by NOESY experiments. The ESI/FAB mass spectra were recorded at RSIC, Lucknow, India, on JEOL SX 102/DA-6000 mass spectrometer data system using Argon/Xenon (6 kV, 10 mA) as the FAB gas. HRMS mass spectra were recorded at IIT-Kanpur, India, on Waters Q-Tof Premier Micromass HAB 213 mass spectrometer using capillary voltage 2.6–3.2 kV. The samples dissolved in suitable solvents were introduced into the HRMS source through a syringe pump at the rate of 3 μl/min. IR spectra were recorded on a Bruker FT-IR Vector 22 model from 4000 to 400 cm$^{-1}$. Melting points of compounds synthesized were measured with the help of JSGW melting apparatus and all the values were uncorrected. Elemental analyses were performed on a Thermoquest CE instrument CHN elemental analyser (Model EA/10). Con A was purchased from Sigma Aldrich and its purity was checked by running an SDS gel and was used without further purification. D-(+)-Mannose was obtained from SISCO Research laboratories PVT. Ltd., Mumbai India; perchloric acid from S.d. Fine-chem limited, Mumbai; acetic anhydride, acetic acid, and urea from S.d. Fine-chem limited; pentaacetyl mannose synthesized in laboratory; boron trifluoride etherate from Spectrochem Pvt. Ltd; tris(2-aminoethyl)amine, 1,4-diaminobutane, 1,3-propanediol, and 2-ethyl-2-(hydroxymethyl)-1,3-propanediol obtained from Lancaster Synthesis, England. All the solvents used were distilled and dried according to established procedures. Images of Con A–mannosylated derivative complexes were taken by an AFM (Molecular Imaging) operating under the Acoustic AC mode (AAC), with the aid of a cantilever (NSC 12(c) from MikroMasch).

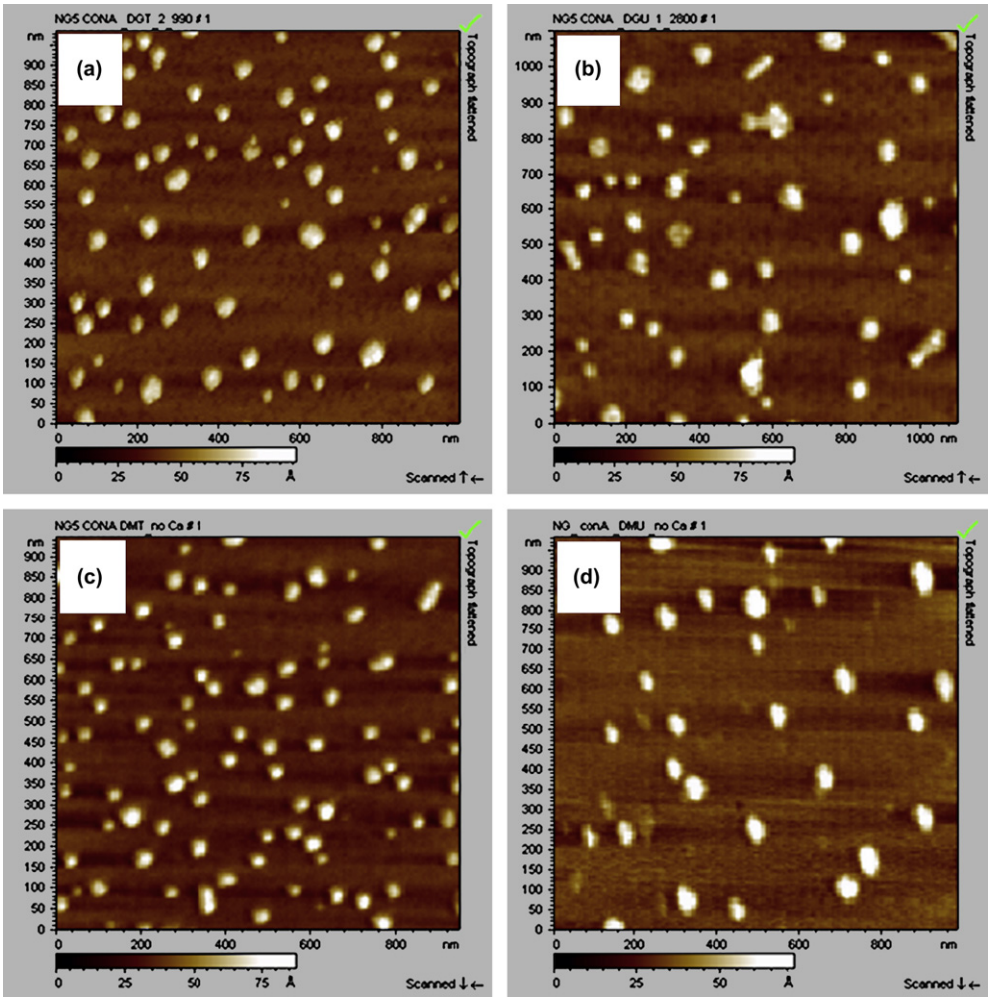



**Figure 6.** AFM images of Con A with (a) *N,N′*-di-α-D-galactopyranosylurea (**1a**), (b) tris[*N,N*-α-D-galactopyranosyl-2-aminoethyl]amine (**2a**), (c) **1**, without $Ca^{2+}$ and $Mn^{2+}$, and (d) **2**, without $Ca^{2+}$ and $Mn^{2+}$.

## 4.2. Synthesis

### 4.2.1. *N,N′-Di-α-D-mannopyranosylurea (**1**)*[16]

A mixture of D-(+)-mannose (1.0 g, 5.5 mmol) and urea (0.13 g, 2.2 mmol) in the presence of catalytic amount of boric acid (0.013 g) in acetic acid (1 ml) was stirred at 85–95 °C until dissolved. Pressure was reduced and the solvent was distilled at the rate of about 0.1 ml/min. Acetic acid (1 ml) was added periodically after every 10 min to maintain constant volume. The reaction was completed in 4 h. The product precipitated was washed with diethyl ether to remove excess acetic acid and it was recrystallized as a light brown powder by methanol water in ratio 100:1. Yield 0.41 g, 38%; decomposition above 300 °C (lit. >300 °C);[16] $\nu_{max}$ (KBr, $cm^{-1}$) 3399 (br d, N–H str), 2921 (m, O–H str), 1645 (s, amide I), 1568 (s, amide II), 1410 (w, C–N); $^1$H NMR (400 MHz, DMSO, 25 °C, TMS) $\delta$ ppm 7.16 (2H, d, NH, $J$=9.76 Hz), 4.89 (d, H-1, $J$=9.28 Hz), 4.74–4.71 (m), 4.64 (d, $J$=5.12 Hz), 4.4–4.37 (t, $J$=5.64 Hz, 5.84 Hz), 3.64–3.24 (m, sugar), 2.5 (OH, mannose); $^{13}$C NMR (100 MHz, $D_2O$, 25 °C, TMS) $\delta$ ppm 79.61 (2×CH), 78.16 (2×CH), 74.30 (2×CH), 71.34 (2×CH), 67.4 (2×CH), 61.86 (2×CH), 158.91 (CO); *m/z* (HRMS) calculated $[M+Na]^+$ 407.1272, found 407.1276.

### 4.2.2. *N,N′-Di-α-D-galactopyranosylurea (**1a**)*[16]

The mixture of D-(+)-galactose (1.0 g, 5.5 mmol) and urea (0.13 g, 2.2 mmol) in presence of catalytic amount of boric acid (0.013 g) in acetic acid (1 ml) was stirred at 85–95 °C until dissolved. Pressure was reduced and the solvent was distilled at the rate of about 0.1 ml/min. Acetic acid (1 ml) was added periodically after every 10 min to maintain constant volume. The reaction was completed in 4 h. The precipitate of *N,N′*-di-α-D-galactopyranosylurea (**1a**) formed in the reaction was washed with diethyl ether and recrystallized from methanol and water in ratio 100:1. Yield 0.20 g, 19%; light brown powder; decomposition above 190 °C (lit. value 195 °C);[16] $\nu_{max}$ (KBr, $cm^{-1}$) 3424 (br d, O–H str), 2924 (s, O–H str), 2855 (w, O–H str), 1663 (s, amide I), 1569 (s, amide II), 1446 (w, C–N); $^1$H NMR (400 MHz, DMSO, 25 °C, TMS) $\delta$ ppm 6.1 (2H, d, NH, $J$=2.14 Hz), 4.89 (d, H-1, $J$=1.28 Hz), 4.73–4.22 (m, sugar), 3.74–3.14 (m, sugar), 2.5 (OH, mannose); $^{13}$C NMR (100 MHz, $D_2O$, 25 °C, TMS) $\delta$ ppm 78.5 (2×CH), 78.1 (2×CH), 74.4 (2×CH), 71.15 (2×CH), 66.84 (2×CH), 61.4 (2×CH), 155.6 (CO). *m/z* (HRMS) calculated $[M+Na]^+$ 407.1272, found 407.1272.

### 4.2.3. *Tris[N,N-α-D-galactopyranosyl-2-aminoethyl]amine (**2a**)*

Tris(2-aminoethyl)amine (0.26 g, 1.79 mmol) was dissolved in anhydrous methanol (20 ml) and the mixture was stirred at room temperature. D-(+)-Galactose (1.0 g, 5.5 mmol) was added to this solution and the resulting solution was stirred under a nitrogen atmosphere, for 16 h, before storing the reaction mixture at 4 °C for 24 h. The solution was filtered and evaporated under vacuum to yield tris[*N,N*-α-D-galactopyranosyl-2-aminoethyl]amine (**2a**) as a crystalline white powder (0.69 g, 59%). Mp 51 °C; $\nu_{max}$ (KBr, $cm^{-1}$) 3395 (br d, N–H str), 2924 (m, O–H str), 2854 (w, O–H str), 1657 (w,

N–H bend), 703 (w, N–H wagging); $^1$H NMR (400 MHz, $D_2O$, 25 °C, TMS) $\delta$ ppm 3.79 (3H, d, $J$=2.22 Hz, H-1, H-1′, H-1″), 3.75–3.85 (6H, m, H-6), 3.61–3.55 (3H, m, H-2), 3.48–3.45 (3H, m, H-3), 3.37–3.25 (3H, m, H-4), 3.22–3.18 (3H, m, H-5), 2.68–2.60 (6H, m, 3×$CH_2$N), 2.47–2.58 (6H, m, NH($CH_2$)$_3$); $^{13}$C NMR (100 MHz, $D_2O$, 25 °C, TMS) $\delta$ ppm 97.85 (C-1), 73.58 (CH), 73.57 (CH), 72.67 (CH), 69.52 (CH), 65.02 (CH), 55.89 (3×$CH_2$N), 38.38 (NH($CH_2$)$_3$); $m/z$ (HRMS) calculated $[M+H]^+$ 633.3194, found 633.3196.

*4.2.4. 1,3-Bis[O-α-D-mannopyranosyl]propane-1,3-diol (**3**)*

1,2,3,4,6-Penta-*O*-acetyl-α-D-mannopyranoside (2.0 g, 5.12 mmol) was dissolved in dry dichloromethane (20 ml) and 1,3-propanediol (0.15 ml, 2.05 mmol) was added followed, after 30 min, by the addition of boron trifluoride etherate (4.25 ml, 35.8 mmol) at 0 °C. The reaction mixture was stirred under nitrogen atmosphere at room temperature overnight. After the completion of reaction as judged by TLC, reaction mixture was diluted with 200 ml dichloromethane and the combined organic layer was washed with (200 ml) water four times, and then brine (200 ml) once, after which it was dried over sodium sulfate, filtered, and concentrated to dryness under reduced pressure. The resulting yellow oil was purified using column chromatography on silica gel (ethyl acetate/hexane, 1:1, v/v) to give 1,3-bis[*O*-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl]propane-1,3-diol as a colorless oil. Yield 0.96 g, 51%; $R_f$ 0.3 (ethyl acetate:petroleum ether in 1:1 ratio); $^1$H NMR (400 MHz, $CDCl_3$, 25 °C, TMS) $\delta$ ppm 5.1–5.24 (6H, m, H-2, H-3, H-4), 4.72 (d, $J$=1.4 Hz, H-1), 3.62–3.73 (2H, m, H-5), 4.22–4.14 (2H, m, H-6), 4.08–3.93 (2H, m, H-6′), 3.44–3.48 (4H, m, $OCH_2$), 3.55–3.05 (2H, m, $OCH_2$), 2.09 (3H, s, OAc), 2.03 (3H, s, OAc), 1.98 (3H, s, OAc), 1.92 (3H, s, OAc), 1.75–1.89 (2H, m, $CH_2$ merged with acetyl peaks). This was directly used in the subsequent conversion without further purification.

1,3-Bis[*O*-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl]propane-1,3-diol (1.2 g, 1.66 mmol) was dissolved in 100 ml of methanol. Sodium methoxide (0.89 g, 16.6 mmol) was added and the reaction mixture was stirred for an hour at room temperature. The resulting solution was neutralized by Amberlite resin IR 120 column, which was activated prior to use by 2 N HCl. The resulting solution was then concentrated to dryness under reduced pressure to yield 1,3-bis[*O*-α-D-mannopyranosyl]propane-1,3-diol (**3**) as a colorless oil (0.46 g, 71%); $\nu_{max}$ (KBr, cm$^{-1}$) 3391 (br d, O–H str), 2935 (w, O–H str), 2831 (m, O–H str), 1132 (m, C–O–C str), 1063 (s, C–O–C str); $^1$H NMR (400 MHz, $D_2O$, 25 °C, TMS) $\delta$ ppm 4.71 (2H, d, merged with $D_2O$, H-1a, H-1b), 3.40–3.81 (12H, m, H-2a, H-2b, H-3a, H-3b, H-4a, H-4b, H-5a, H-5b, H-6a, H-6b, 4H-[($CH_2$)$_3$]), 1.7–1.8 (2H, m, ($CH_2$)$_3$); $^{13}$C NMR (100 MHz, $D_2O$, 25 °C, TMS) $\delta$ ppm 100.42 (2×CH), 73.36 (2×CH), 71.31 (2×CH), 70.6 (2×CH), 67.40 (2×CH), 66.53 ($CH_2$), 61.36 ($OCH_2$), 34.03 ($CH_2$); $m/z$ (HRMS) calculated $[M+Na]^+$ 423.1479, found 423.1476.

*4.2.5. Tris[O-α-D-mannopyranosyl]-2-ethyl-2-(hydroxymethyl)-1,3-propanediol] (**4**)*

1,2,3,4,6-Penta-*O*-acetyl-α-D-mannopyranoside (2.0 g, 5.12 mmol) was dissolved in distilled dichloromethane (20 ml) and 2-ethyl-2-(hydroxymethyl)-1,3-propanediol (0.19 g, 1.46 mmol) was added followed, after 30 min, by the addition of boron trifluoride etherate (4.25 ml, 35.8 mmol) at 0 °C. The reaction mixture was stirred under nitrogen atmosphere at room temperature overnight. After the completion of reaction as judged by TLC, reaction mixture was diluted with 200 ml dichloromethane and the combined organic layer was washed with (200 ml) water four times, and then brine (200 ml) once, after which it was dried over sodium sulfate, filtered, and concentrated to dryness under reduced pressure. The resulting yellow oil was purified using column chromatography on silica gel (ethyl acetate/petroleum ether, 1:1, v/v) to give tris[*O*-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl]ethane-1,2-diol as a colorless oil Yield 0.99 g, 53%; $R_f$ 0.3 (ethyl acetate/petroleum ether in 1:1 ratio); $^1$H NMR (400 MHz, $CDCl_3$, 25 °C, TMS) $\delta$ ppm 5.27–5.12 (9H, m, H-2, H-3, H-4),4.73 (3H, d, $J$=5.4 Hz), 4.26–4.18 (3H, m, H-6), 4.05–3.99 (3H, m, H-6′), 3.97–3.86 (3H, m, H-5), 3.65–3.60 (2H, m, $OCH_2$), 3.41–3.28 (4H, m, $OCH_2$), 2.09 (3H, s, OAc), 2.03 (3H, s, OAc), 1.98 (3H, s, OAc), 1.92 (3H, s, OAc), 0.85 (3H, t, $J$=7.6 Hz, $J$=7.32 Hz), 1.43–1.33 (2H, $CH_2$). This was then directly used without further purification for subsequent conversion.

1,2,3-Tris[*O*-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl]-2-ethyl-2-(hydroxymethyl)-1,3-propanediol (1.3 g, 1.15 mmol) was dissolved in 100 ml of MeOH. Sodium methoxide (0.62 g, 11.5 mmol) was added and the reaction mixture was stirred for an hour at room temperature. The resulting solution was neutralized by Amberlite IR 120. The resin was then filtered off under gravity and the resulting solution concentrated to dryness in vacuo to yield tris[*O*-α-D-mannopyranosyl]-2-ethyl-2-(hydroxymethyl)-1,3-propanediol (**4**). Yield 0.42 g, 69%; $\nu_{max}$ (KBr, cm$^{-1}$) 3399 (br d, O–H str), 2926 (br d, O–H str), 1134 (m, C–O–C str), 1062 (s, C–O–C str); $^1$H NMR (400 MHz, $D_2O$, 25 °C, TMS) $\delta$ ppm 3.98 (H, d, merged with $D_2O$, H-1a, H-1b, H-1c), 3.77–3.37 (15H, m, H-2, H-3, H-4, H-5, H-6, 4H-($CH_2$)$_3$), 3.21–3.16 (6H, m, ($OCH_2$)$_3$), 1.2–1.3 (2H, m, $CH_2$), 0.69 (2H, t, $CH_3$); $^{13}$C NMR (100 MHz, $D_2O$, 25 °C, TMS) $\delta$ ppm 100.49 (3×CH), 72.90 (3×CH), 70.87 (3×CH), 70.21 (3×CH), 68.41 (3×CH), 66.98 (3×CH), 61.02 (3×$CH_2O$), 49.0 (C), 23.4 ($CH_2$), 6.96 ($CH_3$); $m/z$ (HRMS) calculated $[M+Na]^+$ 643.2426, found 643.2421.

### 4.3. Preparation of samples for AFM

All the measurements were performed in the HEPES buffer prepared in double distilled water (10 mmol, pH 7.4). Carbohydrate solution (10 μl, 100 μM) was added to protein solution (10 μl, 0.5 mg/ml) and the volume was made up to 40 μl with a freshly prepared solution of HEPES containing $MnCl_2$ and $CaCl_2$ (1 mM final concentration). These solutions were then incubated for 1 h at 37 °C. Each sample was diluted 50-fold with water and a 20 μl aliquot was deposited on the freshly cleaved mica surface. The sample was dried under mild vacuum and imaged in air with an atomic force microscope (Molecular Imaging) operating under the Acoustic AC mode (AAC), with the aid of a cantilever (NSC 12(c) from MikroMasch). The force constant was 0.6 N/m; the resonant frequency was 150 kHz. The images were taken in air at room temperature with scan speed of 1.5–2.2 lines/s. The data acquisition was done using PicoScan5 software; the data analysis was done with the aid of visual SPM.

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## Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2008.05.055.

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