Unprecedented Inhibition of Glycosidase-Catalyzed Substrate Hydrolysis by Nanodiamond-Grafted $O$-Glycosides

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We report herein the unprecedented finding that $\alpha$-$O$-glucosides and also $\alpha$-$O$-mannosides, when conjugated on nanodiamond particles (ND) are not only stable towards the hydrolytic action of the corresponding matching glycosidases, but are also endowed with the ability to inhibit them. Moreover, conjugation of the $O$-glycosides to ND (glyco-ND) sees them transformed into inhibitors of mismatching enzymes (for which they do not serve as substrates even when in their monovalent, free form). The effects of the glyco-NDs have been demonstrated on a panel of commercial glycosidases and the inhibition found to be competitive and reversible and not to be related to any denaturation of enzymes by the ND-conjugates. Values for $K_i$ in the low micromolar have been measured for certain glyco-ND (for example, a $K_i$ value of $5.5 \pm 0.2 \, \mu M$ was measured for the glyco-ND against the $\alpha$-glucosidase from baker’s yeast) and found to depend on both the identity of the enzyme and the glyco-ND. The latter $K_i$ value compares well with that obtained for the natural glucosidase inhibitor, 1-deoxynojirimycin ($K_i$ of 25 $\mu M$ against the $\alpha$-glucosidase from baker’s yeast under identical assay conditions). The monovalent control $O$-glycosides were hydrolysed efficiently by the appropriate glycosidase. Glyco-ND bearing 50% loading of $O$-glycoside as well ND conjugated with both $O$-glucosides and $O$-mannosides (mixed) have also been assayed and shown also to inhibit the panel of glycosidases with potencies and selectivities different from those recorded for the 100% loaded ND and also from one another. The impact on factors such as glycotope density and heteromultivalency on inhibition is reminiscent of that typically encountered in carbohydrate-lectin recognition events. The abilities of the glyco-ND to bind, cross-link and aggregate concanavalin A, a lectin known to recognize both $\alpha$-$O$-$D$-mannosides and $\alpha$-$O$-$D$-glucosides were assessed by a range of methods including an enzyme-linked lectin assay (ELLA), a two-site sandwich ELLA and a turbidimetry assay, respectively and indeed seen to reflect their expected per glycotope affinity enhancements as compared to monovalent controls: the high avidity of the lectin for each respective glycosylated ND particle was consistent with the manifestation of potent multivalent effects driving lectin recognition and binding.
1. Introduction

A considerable amount of effort has been devoted to studying the principles governing the interactions of natural glycans and their synthetic mimetics with glycan-specific protein partners (lectins). This has demonstrated that the multivalent presentation of a particular glycocone on an appropriate scaffold (i.e. cell-membrane, dendrimer, polymer, nanoparticle, etc.) can affect significantly its potency and selectivity of binding with a given target protein, relative to that of a monovalent counterpart. Indeed, the promise that specific lectin-mediated processes (i.e., pathogen recognition, viral entry, tumor migration and metastasis events, etc.) might be effectively modulated with a properly tailored multivalent ligand continues to see innovative glycoconjugates proposed in the hope of obtaining effective carbohydrate-based therapeutics. In contrast, much less effort has been consecrated to unravelling the principles underpinning interactions of natural or synthetic multivalent glycans with proteins other than lectins, for example with glycosyl hydrolases (glycosidas). Glycosidas constitute a large and important family of enzymes that are responsible for assuring the proper biosynthesis and/or biodegradation of glycoproteins, glycolipids and also proteoglycans - the very entities that serve as ligands for complimentary lectins in vivo.

We herein examine the behaviour of glycosidases towards a series of glycan-conjugated nanodiamonds (glyco-ND). Diamond nanoparticles (also termed nanodiamonds) are among the most promising new carbon based materials for biomedical applications. Advantages of ND over other carbon-based materials such as fullerenes and carbon nanotubes include their complete inertness, optical transparency, lack of significant cytotoxicity in a variety of cell types as well as their ease of functionalization through a variety of methods depending on ultimate application. Mannose-functionalized ND, for example, has been shown to inhibit yeast-agglutination as well as human bladder-cell adherence by E. coli and most notably to be able to disrupt biofilm formation. Indeed, the usefulness of various ND adds the interpretation of glycans-mediated processes has been substantiated recently in a number of reports. These properties have been determinant in their choice to explore the utility of the inhibition behavior of glyco-ND towards glycosidases.

We report herein the unprecedented finding that monosaccharide substrates of selected glycosyl hydrolases behave as inhibitors of their complementary (matching) enzyme, simply upon being conjugated in a multivalent fashion to an ND edifice. Also striking is the finding that multivalent presentation of a given monosaccharide motif on an ND can select the “switching on” of the inhibition of non-complementary (mismatching) glycosidases in a surface density-dependent manner.

2. Experimental section

2.1. Materials

\(N,N'\)-dicyclohexylcarbodiimide (DCC), 4-dimethyl aminopyridine (DMAP), anhydrous acetonitrile, L-aspartic acid, copper(II) sulphate pentahydrate \((\text{CuSO}_4 \cdot 5\text{H}_2\text{O}, \geq 98\%)\), propargyl alcohol, ethylenediaminetetraacetic acid (EDTA), phenol, sulfuric acid \((\text{H}_2\text{SO}_4)\), sodium ascorbate \(( \geq 98\%)\), L-aspartic acid, methanol \((\text{MeOH})\), dichloromethane \((\text{DCM})\), acetonitrile, ethanol, tert-butanol \((\text{tert-ButOH})\) and sodium methoxide were obtained from Sigma-Aldrich and used without any further purification.

Methyl 4-azidobenzoate solution in tert-butyl methyl ether \(( \geq 95\%)\) and 4-azidobenzoic acid was purchased from TCI Europe, Belgium.

The glycosidases \(\beta\)-glucosidase (from bovine liver, cytosolic), \(\alpha\)-galactosidase (from Aspergillus niger), \(\alpha\)-glucosidase (from green coffee beans), \(\beta\)-glucosidase (from almonds), amyloglucosidase (from Aspergillus niger), \(\alpha\)-glucosidase (maltase, from yeast), isomaltase (from yeast), naringinase (Penicillium decumbens), \(\beta\)-mannanidase (from Helix pomatia) and \(\alpha\)-mannanidase (from jack bean) used in the inhibition studies, as well as the corresponding \(\alpha\)- and \(\beta\)-nitrophenyl glycoside substrates, concanavalin A, horse-radish peroxidase-labelled concanavalin A, yeast mannan and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma Chemical Co. Milli-Q water \((18 \text{ M}\Omega)\) was used for all experiments. Hydroxylated diamond \((\text{ND-OH})\) particles were obtained from the International Technology Centre, Raleigh, NC, USA. All reagents and solvents were used without further purification unless stated.

2.2. Synthesis of mannanside (1) and glucoside (2) (monovalent substrates)

2.2.1. Methyl 4-[(\(\alpha\)-D-mannopyranosyl)oxy)ethyl]-1H-1,2,3-triazol-1-yl]-benzoate (1): Ligand (1) was synthesized according to the literature with some modifications. A mixture of propargyl-mannose \((45 \text{ mg}, 0.21 \text{ mmol}, 1.0 \text{ eq})\), \(0.5 \text{ M}\) of methyl 4-azidoazidobenzoate solution in tert-butyl methyl ether \((619 \mu\text{L}, 0.31 \text{ mmol}, 1.5 \text{ eq})\), \(13 \text{ mg}, 0.05 \text{ mmol}, 0.25 \text{ eq}\) copper(II) sulphate pentahydrate \((\text{CuSO}_4 \cdot 5\text{H}_2\text{O})\) and sodium ascorbate \((18 \text{ mg}, 0.11 \text{ mmol}, 0.5 \text{ eq})\) was dissolved in degassed tert-ButOH/H2O \((1:1, 8 \text{ mL})\) under nitrogen. After stirring for 1 day at 50 °C, the solvents were removed in vacuum and the crude mixture was purified by silica gel chromatography \((\text{DCM}/\text{MeOH} 9:1)\) to yield ligand (1) as a colorless solid \((49 \text{ mg}, 77\%)\); \[^{1}H\text{ NMR} (75 \text{ MHz, CD}_{3}\text{OD}): \delta 167.4 \text{(C=O), 146.7 (C8), 141.6, 132.3 (s), 131.0 \text{(Ar), 123.6 (C9), 121.2 (s) (Ar), 101.1 (C1), 75.1 (C5), 72.5 (C3), 72.1 (C2), 68.7 (C4), 63.1 (C6), 60.8 (C7), 52.9 (OCH3)}\text{(s)}; \text{HRMS (ESI+): calcd. for C}_{21}\text{H}_{27}\text{N}_{3}\text{O}_{8} \text{[M + Na}^{+}] 418.1221; found 418.1221; HPLC (C4, 254 nm): \text{t}_{R} = 10.065 (93.9\%)\) (Supporting information Figure S1).

2.2.2. Methyl 4-[(\(\alpha\)-D-glucopyranosyl)oxy]ethyl]-1H-1,2,3-triazol-1-yl]-benzoate (2): A mixture of propargyl-glucose \((35 \text{ mg}, 0.16 \text{ mmol}, 0.5 \text{ M}\) of methyl 4-azidoazidobenzoate solution in tert-butyl methyl ether \((482 \mu\text{L}, 0.24 \text{ mmol})\), \(0.5 \text{ M}\) methyl 4-azidoazidobenzoate \((10 \text{ mg}, 0.04 \text{ mmol})\) and sodium ascorbate \((14 \text{ mg}, 0.09 \text{ mmol}, 0.5 \text{ eq})\) was dissolved in degassed tert-ButOH/H2O \((1:1, 6 \text{ mL})\) under nitrogen. After stirring for 1 day at 50 °C, the solvents were removed in vacuo and the crude product was purified on silica gel chromatography \((\text{DCM}/\text{MeOH} 9:1)\) to yield ligand (2) as a colorless solid \((49 \text{ mg}, 77\%)\); \[^{1}\text{H} \text{NMR} (75 \text{ MHz, CD}_{3}\text{OD}): \delta 167.4 \text{(C=O), 146.9 (C8), 141.6, 132.3 (s), 131.6 (Ar), 123.5 (C9), 121.2 (s) (Ar), 99.9 (C1), 75.1 (C5), 74.1 (C3), 73.5 (C2), 71.8 (C4), 62.7 (C6), 61.6 (C7), 52.9 (OCH3); HRMS (ESI+): calcd. for C_{21}H_{27}N_{3}O_{8} \text{[M + Na}^{+}] 418.1221; found 418.1220; HPLC (C4, 254 nm): \text{t}_{R} = 9.525 (98.8\%)\) (Supporting information Figure S1).

2.3. Modification of diamond nanoparticles

2.3.1. Azide-terminated ND particles (ND-N3): The functionalization of the ND nanoparticles was carried out essentially...
as described previously for Man-ND.\textsuperscript{18} 4-Azidobenzoic acid (0.20 mmol), DCC (0.22 mmol) and DMAP (0.066 mmol) were dissolved in 5 mL anhydrous acetonitrile. A suspension of ND-OH particles (10 mg) in anhydrous acetonitrile (5 mL) was added and the mixture stirred at room temperature for 24 h under nitrogen. The formed ND-N\textsubscript{3} particles were isolated by centrifugation at 10,000 rpm, purified through four consecutive wash/centrifugation cycles at 10,000 rpm with acetonitrile and ethanol, and finally oven dried at 50 °C for 24 h.

2.3.2. Fabrication of Man-, Glc and Glc/Man-ND (Multivalent Particles): ND-N\textsubscript{3} particles (10 mg) were dispersed in water (10 mL) and sonicated for 30 min. The “click” reaction was performed by addition of an alkyne carbohydrate (2 mM) followed by CuSO\textsubscript{4}·5H\textsubscript{2}O (200 μM) and L-ascorbic acid (300 μM) and subsequent stirring of the resulting suspension for 24 h at room temperature. The crude sugar-conjugated nanoparticles were isolated by centrifugation at 10,000 rpm. In order to remove residual copper a first cleaning step protocol consisting of three washing/centrifugation cycles with EDTA (1 mM solution in water) was implemented. A second cleaning protocol involving three consecutive washing/centrifugation cycles at 10,000 rpm with a water-ethanol mixture was also carried out. The resulting particles were finally oven-dried at 50 °C for 24 h prior to use.

For the Glc-ND (50 %) and Man-ND (50 %), the appropriate sugar (1 mM) and propargyl alcohol (1 mM) were mixed prior to being subjected to the “click” with ND-N\textsubscript{3} (10 mg) and processed as described above. Similarly, for the Glc/Man-ND (“mixed”), propargyl glucose and propargyl mannose (1 mM) were mixed with tND-N\textsubscript{3} (10 mg) prior to being subjected to “click” conditions and processes as described above.

2.4 Determination of the carbohydrate loading: A calibration curve for carbohydrate concentrations in solution was established.\textsuperscript{18} For this, a phenolic aqueous solution (5 wt%, 60 µL) and concentrated H\textsubscript{2}SO\textsubscript{4} (900 µL) was added to an aqueous carbohydrate solution (60 µL), stirred for 10 min and then an absorption spectrum of the mixture was recorded (Perkin Elmer Lambda 950 dual beam) against a blank sample (without carbohydrate). The absorbance of the solution was measured at two wavelengths: λ\textsubscript{1}=495 and λ\textsubscript{2}=570 nm and the absorbance difference (A\textsubscript{495}− A\textsubscript{570}) plotted against the concentration of the corresponding carbohydrate. The quantity of surface-linked carbohydrate on glyco-ND was determined with a 60 µL aliquot of the corresponding ND particles solution in water, which was treated with phenol/H\textsubscript{2}SO\textsubscript{4} following the same protocol described above. Propargyl alcohol-terminated ND particles were subjected to the identical procedure and served as a blank sample.

2.5. Instrumentation

2.5.1. Fourier transform infrared (FTIR) spectroscopy: FTIR spectra were recorded using a Perkin-Elmer Spectrum One FT-IR spectrometer with a resolution of 4 cm\textsuperscript{-1}. Dried ND powder (1 mg) was mixed with KBr powder (100 mg) in an agate mortar. The mixture was pressed into a pellet under 10 tons load for 2-4 min and the spectrum was recorded immediately. Sixteen accumulative scans were collected. The signal from a pure KBr pellet was subtracted as a background.

2.5.2. Particle size measurements: ND suspensions (20 µg.mL\textsuperscript{-1}) in water were sonicated. The particle size of the ND suspensions was measured at 25°C using a Zetaviz Nano ZS (Malvern Instruments S.A., Worcestershire, U.K.) in 173° scattering geometry and the zeta potential was measured using the electrophoretic mode.

2.5.3. NMR data: \textsuperscript{1}H NMR spectra were recorded with a Bruker Advance 300 MHz spectrometer using the deuterated solvent as the lock and TMS as an internal standard. Chemical shifts (δ) and coupling constants (J) are expressed in ppm and Hertz (Hz), respectively.

2.5.4. Transmission electron microscopy (TEM): TEM measurements were performed on a FEI Tecnai G2-F20 microscope.

2.5.5. X-ray photoelectron spectroscopy (XPS): XPS measurements were performed in a Specs analysis chamber, equipped with a monochromatized Al K\textsubscript{α} X-ray source (hv = 1486.74 eV) and a Phoibos 150 mm radius hemispherical electron energy analyzer. The analyzer (constant) pass energy was set to 100 eV for survey spectra and at 20 eV for high resolution scans, with an estimated total (source + analyzer + core hole width) resolution of 0.85 eV for the N 1s spectra. The pressure in the analysis chamber was in 10\textsuperscript{-8} Pa vacuum range, and an electron flood gun operating at 1 eV energy and 100 µA electron current was used to ensure sample neutralization. Electrons are recorded at normal emission in “Large Area Mode” of the Phoibos analyzer. The XPS data presented are deconvoluted by using mixed Lorentz/Gauss profiles with the CasaXPS software.

2.5.6. GC-FID analysis: GC-FID was carried out using an Agilent 7820A chromatograph with an EPC injector fitted with a cross-linked 5% phenyl-dimethylsiloxane column (HP-5; 30 m x 320 µm x 0.25 µm). Operating conditions were: injection port temperature 310 °C; splitting ratio 25:1; injection volume 1 µL of derivatized samples; column oven temperature programmed from180 to 310 °C at 5 °C min\textsuperscript{-1}, with a 25 min hold at 310 °C; carrier gas helium (constant flow at 1.2 mL min\textsuperscript{-1}); detector port temperature 310 °C. Total acquisition time was 45 min. The identity of d-mannose (elution time 4.85/5.1 min) and d-glucose (elution time 4.9/5.2) was confirmed by comparison with the GC chromatograms of authentic samples. Calibration curves for quantitative determination were built by using a range of concentrations, from which response factors relative to the I.S. (elution time 8.3/8.5 min) were determined.

2.6. Bioassays

2.6.1. Inhibition assay to determine the interactions of Man-ND, Glc-ND, Man-ND (50 %), Glc-ND (50 %) and Glc/Man-ND with glycosidases: Inhibitory potencies were determined by spectrophotometrically measuring the residual hydrolytic activities of the glycosidases against the respective α- (for β-glucosidase/β-galactosidase from bovine liver) or p-nitrophenyl α- or β-d-glycopyranoside, in the presence of the corresponding iminosugar derivative. Each assay was performed in phosphate or phosphate-citrate (for α- or β-mannosidase or amyloglucosidase) buffer at the optimal pH for each enzyme. The K\textsubscript{m} values for the different glycosidases used in the tests and the corresponding working pH are listed herein: β-glucosidase (bovine liver), K\textsubscript{m} = 2.0 mM (pH 7.3); α-glucosidase (yeast), K\textsubscript{m} = 0.35 mM (pH 6.8); β-glucosidase (almonds), K\textsubscript{m} = 3.5 mM (pH 7.3); α-galactosidase (coffee beans), K\textsubscript{m} = 2.0 mM (pH 6.8); amyloglucosidase (Aspergillus niger), K\textsubscript{m} = 3.0 mM (pH 5.5); naringinase (Pennisetum decumbens), K\textsubscript{m} = 2.7 mM (pH 6.8); β-mannosidase (Helix pomatia), K\textsubscript{m} = 0.6 mM (pH 5.5); α-mannosidase (jack bean), K\textsubscript{m} = 2.0 mM (pH 5.5). The reactions were initiated by addition of enzyme to a solution of the substrate in the absence or presence of various concentrations of inhibitor. After the mixture was incubated for 10-30 min at 37 °C the reaction was quenched by addition of 1 M NaCO\textsubscript{3}. The absorbance of the resulting mixture was determined at 405 nm or 505 nm. The K\textsubscript{i} value and
2.6.2. Procedures to monitor the stability of glyco-ND and the monovalent reference ligand (1) and (2) towards α-mannosidase and α-glucosidase: The susceptibility of glyco-ND and the monovalent α-D-mannopyranose and α-D-glucopyranyl glycosides (1) and (2) towards α-mannosidase and α-glucosidase hydrolysis was examined by incubating each conjugate with the corresponding enzyme at 37 °C under identical conditions to those above described for determination of the inhibition constants during 1 h and monitoring the formation of free mannose or glucose by gas chromatography (GC). For GC analysis, the samples were subjected to an oximation-trimethylsilylation protocol as reported in Ref.31 Briefly, immediately after quenching, the samples were freeze-dried. To 15-20 mg of each sample, deionized water (1 mL) was added. To 100 μL of the resulting solution was then added 100 μL of internal standard (I.S.; 4 mg mL⁻¹ phenyl β-D-glucopyranoside in acetone-water 1:9, v/v) and the final solution was evaporated to dryness at 60 °C (drying oven). The residue was treated with 1 mL of a solution of hydroxylamine in pyridine (20 mg mL⁻¹) at 60 °C over 50 min with mixing at intervals. Hexamethyldisilazane (200 μL) and trimethylchlorosilane (100 μL) were then added, and the reaction mixtures were kept at 60 °C over a further 40 min period. Formation of a white precipitate was observed during this operation, which was separated by centrifugation (13,000 rpm, 5 min) before injection in the GC apparatus. It is worth noting that following oximation-trimethylsilylation derivatization, reducing monosaccharides provides two peaks in the GC chromatograms, corresponding to the syn- and anti-TMS-oximes, whereas the I.S. provides a single peak.

2.6.3. Enzyme-Linked Lectin Assay (ELLA): Nunc-Immuno™ plates (MaxiSorp™) were coated overnight with yeast (Saccharomycetes cerevisiae) mannan at 100 μL/well diluted from a stock solution of 10 μg mL⁻¹ in 0.01 M phosphate buffer saline (PBS, pH 7.3 containing 0.1 mM Ca⁺² and 0.1 mM Mn⁺²) at room temperature. The wells were then washed three times with 300 μL of washing buffer (containing 0.05% (v/v) Tween 20) (PBST). The washing procedure was repeated after each of the incubations throughout the assay. The wells were then blocked with 150 μL/well of 1% BSA/PBS for 1 h at 37 °C. After washing, the wells were filled with 100 μL of serial dilutions of horseradish peroxidase labelled concanavalin A lectin (ConA-HRP) from 10⁻¹ to 10⁻⁷ mg mL⁻¹ in PBS, and incubated at 37 °C for 1 h. The plates were washed and 50 μL/well of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (0.25 mg mL⁻¹) in citrate buffer (0.2 M, pH 4.0 with 0.015% H₂O₂) was added. The reaction was stopped after 20 min by adding 50 μL/well of 1 M H₂SO₄ and the absorbances were measured at 405 nm. Blank wells contained citrate-phosphate buffer. The concentration of lectin-enzyme conjugate that displayed an absorbance between 0.8 and 1.0 was used for inhibition experiments. In order to carry out the inhibition experiments, each glyco-ND or the control non-glycosylated ND sample was added in a serial of 2-fold dilutions (60 μL/well) in PBS with 60 μL of the desired ConA-peroxidase conjugate concentration on NuncIon™ (Delta) microtiter plates and incubated for 1 h at 37 °C. The maximum concentration was kept in all cases at 150 μM to prevent aggregation phenomena; below this concentration, no precipitation was observed under the experimental setup. The above solutions (100 μL) were then transferred to the mannan-coated microplates, which were incubated for 1 h at 37 °C. The plates were washed and the ABTS substrate was added (50 μL/well). Color development was stopped after 20 min and the absorbances were measured. IC₅₀ values, assumed to be proportional to the corresponding binding affinities, were calculated from the percentages of inhibition with up to eleven different concentrations of each conjugate sample as follows:

% Inhibition = (A_{no inhibitor} - A_{with inhibitor}) / A_{no inhibitor} x 100

Results in triplicate were used for the plotting the inhibition curves for each individual ELLA experiment. Typically, the IC₅₀ values (concentration required for 50% inhibition of the Con A-yeast mannan association) obtained from several independently performed tests were in the range of ±12%. Nevertheless, the relative inhibition values calculated from independent series of data were highly reproducible.

2.6.4. Two-site ELLA (sandwich assay): Nunc-Immuno™ plates (MaxiSorp™) microtiter plates were coated with yeast mannan and blocked with BSA as described above. Unlabelled (therefore crosslinkable) ConA lectin was then added at 100 μL/well from a stock solution of 5 μg/mL in 0.01 M phosphate buffer (PBS, pH 7.3, containing 0.1 mM Ca⁺² and 0.1 mM Mn⁺²) for 2 h at 37 °C. The synthesized glyco-ND and the hydroxylated-ND negative control were used as stock solutions of 0.15 mmol/mL in PBS. The ligands were added in serial 2- to 10-fold dilutions (50 μL/well) in PBS and incubated at 37 °C. After 1 h, horseradish peroxidase-labeled ConA lectin (50 μL/well of 200-fold dilution of a 1 mg/mL stock solution in PBS, pH 7.3, containing 0.1 mM Ca⁺² and 0.1 mM Mn⁺²) was added to the microtiter plates which were incubated for another hour at 37 °C. The plates were washed with PBS, and 50 μL/well of ABTS (1 mg/4 mL) in citrate-phosphate buffer (0.2 M, pH 4.0 with 0.015% H₂O₂) was added. The reactions were stopped after 30 min by adding 50 mL/well of 1 M H₂SO₄, and the optical density was measured at 405 nm relative to 570 nm (SI Figure S4).

2.6.5. Turbidity Assay: Solutions of the glyco-ND (50 μL) at the appropriate concentration (37.5, 18.7 and 9.3 μM) in PBS were added to a solution of ConA (50 μL; 1 mg mL⁻¹ in PBS, pH 7.3, containing 0.1 mM Ca⁺² and 0.1 mM Mn⁺²). The time-dependent turbidity kinetics was recorded by measuring the absorption coefficient at 490 nm at intervals of 1 min for 35 min. After 15 min, D-mannose was added to the suspensions to have an excess of about 2500-5000 fold relative to the ND yeast sample to check the reversibility of the aggregation (SI, Figure S5, A-C). The initial rate of precipitation (V) was determined by linear fits of the initial portion of the data (SI, Figure S5, D).

2.6.6. Two-Site Competitive Lectin—Glycosidase Enzyme-Linked Lectin Assay: Nunc-Immuno™ plates (MaxiSorp™) microtiter plates were coated with yeast mannan, blocked with BSA as described above and further coated with unlabelled ConA lectin at 100 μL/well of a stock solution of 5 μg/mL in 0.01 M phosphate buffer (PBS, pH 7.3, containing 0.1 mM Ca⁺² and 0.1 mM Mn⁺²) for 2 h at 37 °C. The glyco-ND, used as 75 μM solutions in PBS, were then added (50 μL/well) and incubated at 37 °C. At this concentration, a classical two-site ELLA (see above) provided optical density values in the range 0.61 to 0.45 (A.U.), which were normalized at 100% crosslinking for the lectin-glycosidase competition experiments. The yeast maltase in serial 2-fold dilutions (50 μL/well) from a stock solution of 40 U/mL in PBS and ConA-HRP lectin (50 μL/well of 100-fold dilution of a 1 mg/mL stock solution in PBS) were added and the microplates were incubated at 37 °C. The plates were washed with PBS and 50 μL/well of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, 1 mg/4 mL) in citrate-phosphate buffer (0.2 M, pH 4.0 with 0.015% H₂O₂) was added. The reactions were stopped after 30
min by adding 50 μL/well of 1 M H₂SO₄. The optical density was then measured at 410 nm relative to 570 nm and plot against maltase concentration (SI Figure S6). Control experiments were conducted to confirm that the enzyme itself did not interact with ConA and that it retained its catalytic activity under the conditions of the assay.

3. Results and Discussion

The behavior of α-mannosidase from jack bean and α-glucosidase from yeast, towards the corresponding O-mannosides and O-glucosides, respectively, when free in solution or upon being grafted on ND, was selected as the primary focus of the present investigation. The latter enzymes are commercially available and are routinely used for ascertaining the potency and selectivity patterns of putative glycosidase inhibitors. Moreover, simple α-mannosides and α-glucosides are accepted as substrates by their respective α-mannosidase and α-glucosidase (matching enzymes). For the sake of completeness, a β-mannosidase, β-glucosidase and also α- and β-galactosidases, none of which act on either α-mannosides or α-glucosides, were included in the evaluation panel (mismatching enzymes). α-O-Mannoside- and α-O-glucoside-grafted-ND (Man- and Glc-ND, respectively) as well as particles featuring 50% of the maximal surface loading of either sugar (Man-ND (50%) and Glc-ND (50%)) were targeted for evaluation (Figure 1). In addition, mixed ND comprising equal proportions of both α-D-mannopyranoside and α-D-glucopyranoside moieties on their surface were also fabricated (Glc/Man-ND) for study.

3.1. Preparation and characterisation of ND-conjugates and O-glycoside monomers

The strategy for the preparation of all sugar-conjugated ND studied herein involved the “click” reaction between azide-terminated particles (ND-N₃) and the appropriate propargylated partner(s) in the presence of CuSO₄/L-ascorbic acid as catalyst, as previously described (Figure 1A). The successful integration of glycans onto the ND surface was confirmed by XPS and FTIR analysis (Supporting Information, Figure S2A, B).

A representative transmission electron microscopy (TEM) image of Man-ND (Figure 1B) reveals the presence of spherical particles with a mean diameter of 12 ± 4 nm. The data was obtained from an analysis of several hundreds of NPs. The surface-modified layer is not visible in the TEM due to its high transparency to the electron beam. The calculated hydrodynamic diameter of glycans-modified ND is a composite value as they partially aggregate in solution (Table 1). The value however remains unchanged over days, indicating that the conjugates have good colloidal stability in aqueous media. For a complete set of TEMs at each stage of NP functionalization, see Supporting Information, Figure S2C. The complete characterisation data and physico-chemical properties, including the particle diameter and zeta potential of all fabricated particles are summarized in Table 1. The total amount of sugar conjugated to a given particle was quantified using the classical phenol-sulphuric acid method. Indeed, the role of ligand density in glycochemistry is known to have a substantial effect on binding and ligand density on nanoparticles is known to change drastically the interactions between substrates and proteins. The analysis confirmed that the 50% loaded particles contained half the quantity of sugar present on the 100% loaded NPs (Table 1). It has to be noted that the phenol/sulfuric acid method does not allow discrimination between manno and glucopyranosides, but we make the
reasonable approximation that the mixed ND comprise equal portions of Glc/Man on the surface. The corresponding monovalent α-O-mannoside and α-O-glucoside control conjugates (I) and (2) (Figure 1A) required for the study were synthesised by the Cu(I)-catalysed “click” reaction of the appropriate propargyl α-glycoside with 4-azidobenzoic acid methyl ester and proceeds smoothly.

<table>
<thead>
<tr>
<th>ND scaffold</th>
<th>Hydrodynamic diameter (nm)</th>
<th>Zeta potential (mV)</th>
<th>Sugar loading (μg mg−1 ND)</th>
<th>Sugar loading (gycans/ND)</th>
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<td>79 ± 13</td>
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<td>Man-ND</td>
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<td>96 ± 7</td>
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<tr>
<td>Glc-ND</td>
<td>145 ± 3</td>
<td>24.7 ± 0.2</td>
<td>113 ± 5</td>
<td>(50±21) ×10^2</td>
</tr>
<tr>
<td>Glc-Man-ND</td>
<td>124 ± 12</td>
<td>27.0 ± 0.2</td>
<td>110 ± 5</td>
<td>(49±20) ×10^2</td>
</tr>
<tr>
<td>Man-ND (50%)</td>
<td>101 ± 10</td>
<td>28.0 ± 0.6</td>
<td>50 ± 3</td>
<td>(22±13) ×10^2</td>
</tr>
<tr>
<td>Glc-ND (50%)</td>
<td>92 ± 2</td>
<td>28.1 ± 0.8</td>
<td>55 ± 3</td>
<td>(25±11) ×10^2</td>
</tr>
</tbody>
</table>

Table. Summary of selected physico-chemical properties of ND particles.

3.2. Subjection of O-glycoside–ND conjugates and monomers to the hydrolytic action of glycosidases

As expected the monovalent analogs (1) and (2) are hydrolysed by their appropriate partner enzymes, namely α-mannosidase or α-glucosidase, respectively, but remain inert to the hydrolytic action of all other glycosidases tested (gas chromatography monitoring, see supporting information Figure S3).

Having established the hydrolytic susceptibility of these O-glycoside monomers free in solution, their behaviour upon being presented multivalently on the ND surface was examined against the same panel of enzymes. In order to get information on the recognition of glyco-ND ligands by Con A, we performed enzyme-linked lectin assay (ELLA) experiments. This test measures the ability of a soluble saccharide to inhibit the association between a labelled lectin (here Con A lectin labelled with horseradish peroxidase, ConA-HRP) and a ligand immobilized on the microtiter well (here a yeast mannan). The presence of the relatively large HRP protein label (40 kD) prevents two lectin moieties from approaching each other, resulting in 1:1 binding stoichiometries with the saccharide ligand. In order to discount that the ND scaffold itself might have a bearing on the outcome of the assay, a conjugate featuring hydroxymethyltriazolyl chains (devoid from any glycopote) was synthesized and included in the evaluation experiments as a negative control. Methyl α-d-mannopyranoside and methyl α-d-glucopyranoside were also included as monovalent positive controls.

Remarkably, both Glc-ND and Glc-ND (50%), when treated with the α-glucosidase from baker’s yeast under classical assay conditions, revealed themselves to be completely stable to hydrolysis (the formation of mannose was not detectable by gas chromatography even upon incubation under assay conditions for 24 h at 37 ºC; Figure S3) and instead behaved as competitive inhibitors of the enzyme, with inhibition constant (Ki) values of 22 and 5.5 μM, respectively. A hydroxyl-coated ND-conjugate (ND-OH), devoid from monosaccharide appendages, was prepared by clicking propargyl alcohol and 4-azidobenzoate-modified ND particles (precursors C and ND-N1 in Figure 1A) and used as negative control. Although the low water solubility of ND-OH precluded an exhaustive evaluation, no significant glycosidase inhibition was observed at low mM concentrations, discarding a relevant contribution of the ND scaffold to the enzyme binding process. The Glc/Man-ND also displayed a similarly low Ki value of 1.9 μM (Table 2). Screening inhibitory activities of Glc-ND’s against the amyloligosaccharidase (1,4-α-D-glucan glucohydrolase) from Asp. Niger and the isomaltase (oligosaccharide α-1,6-glucohydrolase) from baker’s yeast, both of which hydrolyse α-glucosidic substrates, established none to be inhibitors of the former enzyme but all to inhibit the activity of the latter with Ki’s of 14.0, 4.3 and 5.5 μM (for Glc-ND, Glc-ND (50%) and Glc/Mann-NDs, respectively). In a parallel series of experiments, Man-ND, Man-ND (50%) and Glc/Mann-ND were screened against α-mannosidase from jack bean. These conjugates are seen to be comparatively poor inhibitors of their target enzyme giving, respectively Ki’s of 517, 295 and 407 μM. Again, as expected the corresponding monomeric O-mannoside (1) behaves as a substrate for the jack bean α-mannosidase being efficiently hydrolysed under standard assay conditions, whereas under the same conditions the corresponding O-mannoside-conjugated ND remains intact.

Although the origin of the inhibitory behaviour of the glyco-ND particles remained to be established, the finding that they do act as such is nonetheless unprecedented. Exposure of glycosidases to a fixed concentration of a glyco-ND for prolonged periods (up to 2 h) far in excess of the assay time saw no observable change in catalytic activity, discounting any possibility that protein denaturation by the glyco-NDs contributed to the observed inhibitory effects. The levels of inhibition observed for these ND-grafted O-glycosides are better appreciated if compared with Ki values displayed by 1-deoxyxojirimycin (DNJ), the archetypical natural glucosidase inhibitor.15–18 DNJ, under identical assay conditions to those used to screen the sugar-grafted ND particles, inhibits baker’s yeast α-glucosidase with a Ki of 25 μM, an order of magnitude higher than the mixed-ND (Ki, 1.9 μM). In addition, the glyco-ND particle behaviour can be significantly different from that observed in other lectin conjugates, which generally exhibit some degree of selectivity, behaving as better inhibitors of certain enzymes than others, with a pattern of selectivity that does not always parallel that observed for DNJ (Table 1). Baker’s yeast isomaltase is inhibited for example by Glc-ND (50%) with a Ki of 4.3 μM and also by DNJ (Ki of 11 μM). In contrast the amyloligosaccharidase from Asp. Niger is strongly inhibited by DNJ (Ki of 2.1 μM), whereas none of the sugar-grafted particles have any inhibitory effect on this particular activity.

In a bid to better understand the inhibitory activity observed for the various O-glycoside-conjugated ND particles, they were subjected to further scrutiny as inhibitors of various mismatching enzymes – those that do not accept either α-O-glucosides or α-O-mannosides as substrates. Thus, the Glc-ND (featuring a configured O-glucosidic units) when screened against the β-glucosidase from bovine liver, was observed to be inhibitory with Ki values of 113 and 44 μM for the Glc-ND and the Glc-ND (50%), respectively and a Ki of 192 μM for the Glc/Mann-ND. The Glc-ND proved a poorer inhibitor of a second β-glucosidase (from almond), giving Ki’s of 359, 169 and 784 μM for the corresponding 100%, 50% and mixed-sugar conjugates, respectively. Further, although neither the Glc-ND or Man-ND show activity as inhibitors of the β-mannosidase from Helix pomatia, at the maximum concentrations tested, the Glc-ND (50%), Man-ND (50 %) and also the mixed-ND particles unexpectedly did, with Ki’s of 55, 75 and 74 μM, respectively (Table 2). We were intrigued by the relatively relaxed inhibitory specificity shown by the Glc-NDs and were curious to establish whether this was limited solely to glycosidases acting on α-d-gluco- or α-d-manno-configured substrates. The Glc-ND were thus tested against two additional activities: the α-galactosidase from green coffee bean and the β-galactosidase from E. coli. Both the Glc-ND and the Glc-ND (50%) were found to
inhibit α- and β-galactosidase with almost identical potency ($K_i$’s in the range 13-33 µM; Table 2). The Glc/MAN-Nd inhibited both galactoside activities but differentially, giving a $K_i$ of 69 µM for the α-galactoside and a value of 268 µM for the β-enzyme (Table 2).

That O-glycoside configurational complementarity is not an absolute prerequisite for effective enzyme inhibition by Glyc-Nds is particularly well borne out by their pronounced inhibition of mismatching glycosidases. This latter observation prompted us to examine the effects of the O-mannoside-grafted ND on the complete panel of activities at our disposal, even though these latter conjugates were seen to exhibit only negligible inhibition of the “matching” mannosidase activity. The screening reveals that both the Man-Nd and the Man-Nd (50%) inhibit the baker’s yeast α-glucosidase activity and very significantly so, with $K_i$’s of 9.4 and 1.3 µM, respectively (Table 2). The β-glucosidase from bovine liver is however more poorly inhibited compared to the α-enzyme, giving $K_i$’s of 223 and 108 µM for the Man-Nd and the Man-Nd (50 %), respectively. In addition, the α- and β-galactosidases from coffee bean and E. coli respectively are also strongly inhibited by the Man-Nd, with $K_i$’s of 33 and 13 µM for the Man-Nd and 21 and 13 µM for Man-Nd (50%), respectively, and to an equivalent extent as that observed for the corresponding Glc-Nd (Table 2).

Table 2. Inhibition constants ($K_i$, µM) for various glyco-ND, against selected commercial glycosidases.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Glc-Nd</th>
<th>Glc-Nd (50%)</th>
<th>Glc/MAN-Nd</th>
<th>Man-Nd</th>
<th>Man-Nd (50%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-glucosidase (baker’s yeast)</td>
<td>22 ± 2</td>
<td>5.5 ± 0.2</td>
<td>1.9 ± 0.1</td>
<td>9.4 ± 0.3</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Amyloglucosidase (Asp. niger)</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Isomaltase (baker’s yeast)</td>
<td>14 ± 0.5</td>
<td>4.3 ± 0.2</td>
<td>5.5 ± 0.2</td>
<td>12.0 ± 0.5</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>α-mannosidase (jack bean)</td>
<td>419 ± 35</td>
<td>222 ± 15</td>
<td>407 ± 30</td>
<td>517 ± 50</td>
<td>295 ± 20</td>
</tr>
<tr>
<td>β-glucosidase (bovine liver)</td>
<td>113 ± 5</td>
<td>44 ± 2</td>
<td>192 ± 5</td>
<td>223 ± 10</td>
<td>108 ± 5</td>
</tr>
<tr>
<td>β-glucosidase (almonds)</td>
<td>359 ± 20</td>
<td>169 ± 10</td>
<td>784 ± 50</td>
<td>323 ± 15</td>
<td>105 ± 5</td>
</tr>
<tr>
<td>β-mannosidase (Helix pomatia)</td>
<td>NI</td>
<td>55 ± 3</td>
<td>74 ± 5</td>
<td>NI</td>
<td>75 ± 4</td>
</tr>
<tr>
<td>α-galactosidase (coffee beans)</td>
<td>31 ± 2</td>
<td>22 ± 2</td>
<td>69 ± 5</td>
<td>33 ± 2</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>β-galactosidase (E. coli)</td>
<td>22 ± 1</td>
<td>17 ± 1</td>
<td>268 ± 25</td>
<td>13 ± 2</td>
<td>13 ± 1</td>
</tr>
</tbody>
</table>

*Inhibition was reversible and competitive in all cases except for Glc-Nd (50%) against yeast α-glucosidase (yeast maltase), for which a mixed-mode inhibition mode was observed. **NI: no inhibition observed at 1 mM.*

It is worthwhile noting that, while the $K_i$ values recorded for glycoside-coated ND do not vary dramatically from one another with sugar loading (100% vs 50%) for the majority of enzymes tested, this trend is seen not to be true for the β-mannosidase from Helix pomatia: for the latter enzyme, the 50% Glc- and 50% Man-Nds as well as the mixed ND are inhibitory, whereas the 100% Glc- and Man-Nds are not.

The inhibition data suggests that the mode-of-recognition of the O-glycoside-grafted particles by a particular glycosidase is quite different from that usually harnessed by that enzyme for monovalent substrate hydrolysis. Non-specific aggregation of the glyco-Nds with the tested glycosidases seems improbable considering the observed inhibition activity profiles. Indeed, monosaccarides are currently used as passivation molecules to avoid unspecfic interactions of nanoparticles with biomolecules in physiological media.96, 37 The impact on factors such as glycopeptide density and heteromultivalency in glycosidase inhibition is more reminiscent of that encountered in carbohydrate-lectin recognition events.37 We were in a position to establish whether or not this was indeed the case and set about evaluating the performance of the various glyco-ND as ligands...
for lectins such as concanavalin A (ConA), a tetravalent protein known to recognize both α-D-mannopyranosides and α-D-glucopyranosides. The abilities of the glyco-ND to bind, cross-link and aggregate ConA as assessed by a range of methods including an enzyme-linked lectin assay (ELLA; Figure 2), a two-site sandwich ELLA and a turbidimetry assay, respectively (see supporting information Figure S4 and Figure S5), were duly investigated and indeed seen to reflect their expected per glycostope affinity enhancements as compared to monovalent controls: the high avidity of the lectin for each respective glycosylated ND particles is consistent with the manifestation of potent multivalent effects driving lectin recognition and binding.

Sandwich-type competitive glycosidase-lectin ELLA

Having established that Glc- and Man-ND behave not only as inhibitors of various glycosidases, but also as ligands of ConA, a newly developed sandwich-type competitive glycosidase-lectin ELLA was implemented in the hope of garnering further insights into the mechanism(s) of enzyme inhibition by the glyco-ND. The experimental setting is an interface composed of a mannan polysaccharide adsorbed on a polystyrene microtiter well, onto which is applied a second layer constituted of the tetrameric, and therefore cross-linkable, lectin ConA (Figure 3A).

We reasoned that, if a glycosidase capable of competing with horseradish peroxidase (HRP)-labelled ConA (HRP-ConA) for binding a given glyco-ND were to be included in the mix, a concentration-dependent decrease in lectin cross-linking would ensue. Moreover, inclusion of an excess of a potent active site-directed inhibitor of the particular glycosidase being tested, would result in an assay that reported indirectly, on the extent to which the active site is implicated in any glyco-ND—enzyme complex. We reasoned too that were the assay to be performed with an inhibitor capable of spanning both the catalytic and aglycone binding sites simultaneously, it would then afford insights into the impact of glyco-ND-aglycone binding on inhibition. (Figure 3A). We hoped that the data taken together, would allow the relative individual contributions of either the active site or any peripheral binding region on a particular enzyme, in glyco-ND recognition to be ascertained and thus provide insights into the possible mode(s)-of-inhibition in play.

The ConA—Glc-ND—HRP-ConA cross-linking inhibition plots for yeast α-glucosidase (yeast maltase) alone or in the presence of the competitive maltase inhibitors nojirimycin 5N,6O-(cyclic carbamate) (3) \( K_i = 2.2 \mu M \)34, 38 and methyl 6-O-[nojirimycin-1-yl 5N,6O-(cyclic carbamate)]-α-D-glucopyranoside (4) \( K_i = 5.5 \mu M \)39 are each depicted in Figure 3B. Both (3) and (4) are active-site directed inhibitors: compound (3), a monosaccharide mimic, binds exclusively at the glucose (-1) site of the enzyme whereas the isomaltose mimic (4) spans simultaneously both the glucose (-1) and the aglycone (+1) sites. In the presence of the monosaccharide-like inhibitor (3), and thus when the glycone site of maltase is occupied, the data indicates that the ability of the enzyme to compete with the lectin for the Glc-ND is only nominally compromised. In contrast, any ability of the glyco-ND to form an ND-maltase complex is seen to be severely perturbed in the presence of the pseudodisaccharide homologue (4) and, consequently, lectin cross-linking is largely unimpeded. Identical trends were recorded for the 50% Glc-, 100% and 50% Man-, and mixed Glc/Mann-ND conjugates in this assay (see Figure S6).

Although the remarkable difference between monovalent and multivalent O-glycosides towards the hydrolytic action of glycosidases revealed herein would appear difficult to reconcile with the typical modes-of-action currently accepted for glycosidases, the evidence of the sandwich type competitive enzyme-lectin ELLA allows a plausible alternative mode-of-inhibition to be put forward for the glyco-ND: one that does not parallel the mode of substrate recognition typical of enzymatic hydrolysis, but that instead implicates the interaction of O-glycoside moieties present in glyco-ND with the aglycone.
binding sites of the target enzymes as one of the primary driving forces underpinning their inhibitory activity. This hypothesis is consistent with the fact that the interaction of a given substrate with the catalytic cleft of a particular enzyme is dependent on the interactions of both its glycone and aglycone constituents. Aglycone-binding sites of glycosidases are known to accept a range of structural motifs, and substrates featuring certain of these motifs have been shown be much more susceptible to enzymatic hydrolysis. Indeed, the exploitation of such aglycone site-interactions in glycosidase inhibitor design was recognized some twenty years ago. Glycosidase inhibitors targeting exclusively the aglycone binding site of glycosidases have been reported but are rare. On the other hand, hybrid compounds in which a judiciously selected aglycone moiety and a glycomimetic inhibitor are combined, have been reported to display improved inhibitory potencies and selectivities for their target enzymes compared with compounds featuring only one of the constituent fragments.

As far as we are aware, prior to the present work there has been no report of the transformation of an O-glycoside substrate of a glycosidase into an inhibitor, simply upon being presented multivalently on a scaffold. The resistance of a number of O-glycoside-supported gold NPs to the hydrolytic action of various glycosyl hydrolases has on the other hand been examined previously, although these glyco-NPs were not reported to be inhibitors of these enzymes. Indeed we are only aware of a single previous study that explored multivalent O-glycosides as glycosidase inhibitors. In that, the large bacterial sialidase from *V. cholerae*, that features a catalytic module flanked by two lectin domains (carbohydrate binding modules or CBMs) was reported to be strongly inhibited by a synthetic polymer featuring multiple *O*-galactosyl units. The latter glycopolymer was proposed to owe its activity to its interaction with the CBMs of the *V. cholerae* sialidase, effectively leading to their “sequestration”, resulting in seriously compromising its ability to hydrolyse multivalent *O*-sialoside substrates. The role of CBMs in catalysis has now been widely examined but as far as we are aware, catalysis by the enzymes explored in the present study does not benefit from the presence of discrete flanking CBMs. However, binding of multivalent glyco-ND to distal non-catalytic domains other than CBMs of glycosidases would be expected also to lead to a reduction in their catalytic efficiency. No such domains have been established for any of the enzymes studied here. Additionally, although the β-galactosidase from *E. coli* features multiple catalytic domains and is thus expected to be susceptible to inhibition by appropriate multivalent constructs, it is nevertheless inhibited by both Glc-ND and the Glc-ND (50%) to the same extent and, moreover, only to the same level as seen for the α-galactosidase from coffee bean which only features a single such domain.

4. Conclusions

We describe in this study the unprecedented finding that α-D-O-glycosides as well as α-D-O-mannosides when grafted multivalently on the surface of ND particles are not only rendered stable towards the hydrolytic action of the corresponding matching glycosidases, but are also endowed with the ability to inhibit these enzymes. Moreover, conjugation of the O-glycosides to ND seems them behaving as inhibitors of enzymes for which they do not serve as substrates even when in their monovalent, free form. Furthermore, the inhibitory potency of a particular glyco-ND edifice towards a given enzymatic activity is demonstrated to be dependent not only on the particular sugar motif grafted (α-D-gluc or α-D-manno) but also on whether the glycosidic moieties are presented in homogeneous displays, or as a mixture of glycotopes on a single particle and additionally varies with their surface density (100 or 50% loading). The data support that inhibition by the glyco-ND cannot be one that is catalytic site-independent as has been previously put forward to rationalize the mode-of-inhibition of iminosugar constructs.

Instead, the data of the ConA—yeast α-glucosidase competitive ELLA support an alternative mode-of-inhibition in which glyco-ND are able to *competitively* inhibit catalytic activity through formation of the corresponding glyco-ND—enzyme complexes by harnessing of interactions with the enzyme aglycone binding sites. That additional modes-of-binding might also be operational for one or more enzymes in the panel tested here, cannot be discounted at this stage and remain to be fully elaborated. However, the distinct preferences for sugar motif recognition, dependence on multivalency, heteromultivalency and architectural parameters of a particular glyco-ND, do parallel closely those previously observed for interactions of multivalent ligands with lectins. The mode-of-inhibition implicating aglycone binding proposed here would support that the modes of interaction of glyco-ND with enzymes and lectins share much in common and begs the question as to whether or not this might also hold true for alternate multivalent carbohydrate analogs.

To the best of our knowledge other O-glycoside-based multivalent constructs—many known to interact potently with lectins—have not yet been evaluated as putative inhibitors of glycosidases, although multivalent iminosugar analogs have been extensively studied in recent years. It is reasonable to expect that the novel phenomenon uncovered here may not be limited solely to NDs but is likely also to be manifested by multivalent constructs derived using other nanoparticles or alternate scaffolds. The present data are limited to α-D-glucosides and α-D-mannosides. The incertitude on the mechanistic aspects makes it inappropriate to speculate about the possibility of other O-glycosides also promoting glycosidase inhibition when presented on appropriate scaffolds.

The findings reported herein promise to impact on our understanding of the mechanisms-of-action of glycosyl hydrolases (and possibly those of other catalytic proteins) and will undoubtedly provide new opportunities for the design of synthetic enzyme inhibitors. The possibility that a multivalent ligand designed to modulate a selected lectin-ligand interaction might show cross-reactivity with one or more glycosidase, would seem to further complicate the development of multivalent compounds as therapeutic agents. Moreover, should any native multivalent glycosidase be shown to inhibit, and thereby modulate, glycosidase action in vivo, this would need to be taken into account when rationalising a wide range of key biological phenomena known to be sensitive to the presence of glycans.

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