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Binding of carbohydrates to solid supports: evaluation of a prototype system

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Mono- and disaccharides were covalently and irreversibly bound to aminopolystyrene beads in good yield by heating in dilute aqueous solution. The degree and stability of sugar binding were determined by chemical and radiochemical methods and the accessibility of the bound sugars was demonstrated by exoglycosidase hydrolysis and by an enzyme-linked lectin-binding assay using Concanavalin A.

Keywords: reducing sugars, immobilization, Amadori rearrangement, aminopolystyrene, immunoassay

Introduction

Complex sugars are widely distributed in nature, commonly as oligosaccharides attached to proteins and lipids. As improved methods for separation and analysis have become available [1, 2], an understanding of their importance in biological function has started to emerge [3]. Often only limited amounts of sugars are available for characterization, and there is a need for sensitive detection methods. Radiochemical labelling provides good sensitivity [4], but immunochemical techniques are potentially more sensitive and discriminating. They have not been routinely used for sugar characterization, partly because of the lack of suitable methods for attachment to solid supports.

The most common method for immobilizing reducing sugars is reductive amination, which involves the formation of a glycosylamine of a reducing sugar and an immobilized primary amine, followed by reduction with sodium cyanoborohydride [5]. The approach is experimentally simple, but requires high concentrations of sugars and long reaction times. The technique is more successful when the amino groups of the polymer are coupled to the aldehyde groups of a periodate-treated sugar [6], but this would not be useful as a general method. Other techniques for immobilization of sugars involve epoxide [7], and vinylsulfone [8] chemistry but these, too, provide only modest coupling efficiency. Another approach, which involves copolymerization of unsaturated sugar derivates [9, 10] is not appropriate.

For immunochemical analysis of sugars, an immobilization method is needed which satisfies the following requirements: (1) high efficiency of coupling using low concentrations of sugar; (2) coupling of both aldoses and 2-acylaminoaldoses; (3) coupling under conditions which do not cause degradation of the sugars; (4) coupling which is stable enough to withstand specific enzymic and chemical treatments; (5) coupling chemistry which permits subsequent removal of the bound sugars.

We report here the use of a prototype functionalized polymer, aminopolystyrene, and a modified coupling procedure, which together satisfy some of these requirements, and which demonstrate chemistry sufficiently general to be transferred to other support matrices. Related chemistry has been described in a recent report [11] of the immobilization of sugars on the hydrophilic polymer, Fractogel, for use in the affinity chromatography of lectins and antibodies.

Materials and methods

Polystyrene beads with specular finish, 3.2 mm diameter, were from the Precision Plastic Ball Co. (USA). Concanavalin A (25 mg ml^{-1} in 4 M sodium chloride), Blocking Reagent for ELISA, 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (ABTS), α-glucosidase (ammonium sulfate suspension, 100 U ml^{-1}), glucose oxidase (Grade II in 4 M sodium chloride, 5000 U ml^{-1}) and horseradish peroxidase (Grade 1) were from Boehringer Mannheim (Germany). Rabbit anti-Concanavalin A was from Sigma Chemical Co. (USA) and sheep anti-rabbit IgG-HRP conjugate was from Silenus (Australia). [U-^{14}C] Glucose (290 mCi $mmol^{-1}$ in 5% ethanol) was from CEA (France), *N*-acetyl [1-^{14}C] glucosamine (60.0 mCi $mmol^{-1}$ in ethanol-water) from NEN Research Products (USA) and BCS scintillant from

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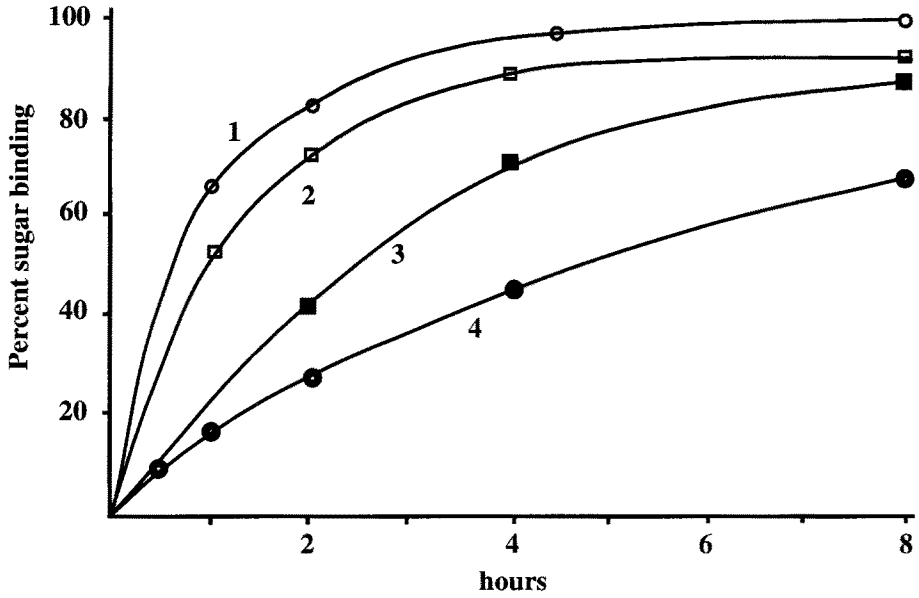

Figure 1. Binding of reducing sugars to aminopolystyrene as a function of time. Curve 1: ^{14}C-glucose (approx. 100 nM) in sodium phosphate buffer (0.1 M, pH 4.3) at 100 °C; curve 2: 1 mM maltose in water at 100 °C, analysed by the phenol-sulfuric acid test; curve 3: 1 mM *N*-acetylglucosamine in water at 100 °C, analysed by the Morgan–Elson test; curve 4: ^{14}C-glucose (approx. 100 mm) in water at 75 °C.

Amersham (UK). Other chemicals were of analytical grade. All phosphate buffers were prepared from the appropriate proportions of sodium dihydrogen phosphate and disodium hydrogen phosphate.

NITRATION OF POLYSTYRENE BEADS

Polystyrene beads (20 g) were added with stirring to a mixture of nitric acid (70%, 25 ml) and sulfuric acid (98%, 30 ml) which had been cooled to 5 °C in an ice bath. Stirring was continued at 5 °C for 4 h. The supernatant was poured off and the cream-coloured beads washed well with water and dried.

REDUCTION OF NITROPOLYSTYRENE BEADS

Stannous chloride dihydrate (15 g) in hydrochloric acid (20 ml) was heated with stirring at 90 °C in an oil bath until dissolved. Nitropolystyrene beads (20 g) were added and stirring continued for 2 h at 90 °C. The supernatant was poured off, and the beads washed several times with hot water, then twice with 1 M NaOH (to break up any chlorostannate salt) then with water and 1 M HCl. The beads were stored at 4 °C under 1 M HCl.

ESTIMATION OF THE DEGREE OF DERIVATIZATION OF BEADS

The beads were shaken for 30 min in 5% sodium hydrogen carbonate solution and washed three times with water and then with ethanol. Four beads were added to each of a series of tubes, 1 ml of an ethanolic solution of 4-nitrobenzaldehyde (4 mM) in acetic acid (1%) added to each, the tubes capped and shaken gently at room temperature for up to 16 h. Aliquots (200 µl) were diluted to 3 ml with ethanol and the absorbance at 275 nm compared with that of the original aldehyde solution.

BINDING OF SUGARS TO AMINOPOLYSTYRENE BEADS

(*a*) *General*

The aminopolystyrene beads were equilibrated with buffer for 1 h or washed well with water before use. In a general procedure, four aminopolystyrene beads were added to a 10 mm capped glass tube and incubated with 300 µl of a sugar solution. The binding of sugars was assessed by determining the amount of sugar remaining in the supernatant. Binding was studied as a function of time and temperature (Fig. 1). In all experiments, blanks were included to determine non-specific binding to beads and glassware.

(*b*) *Radiochemical analysis of supernatants*

After binding, aliquots (2 × 100 µl) of supernatants from duplicate tubes were mixed with scintillant (10 ml) and sugar binding calculated by comparison with counts in aliquots of the original sugar solution.

(*c*) *Chemical analysis of supernatants*

Phenol-sulfuric acid test. Analysis of glucose and maltose was carried out by a modification of the method of Dubois [12]. Aliquots (3 × 75 µl) of supernatant were taken from each tube, mixed well with phenol solution (2.5%, 2 ml) and sulfuric acid (98%, 5 ml) added rapidly. The solutions were allowed to cool to room temperature and read at 490 nm. The sugars remaining in the supernatants were estimated by comparison with standard maltose solutions.

Table 1. Binding of maltose to aminopolystyrene beads.[a]

Detected sugar	*Method of analysis*	*Maltose (nmol)*
Bound maltose	Enzymatic	176
Bound maltose	Chemical	147
Released glucose	Enzymatic[b]	76

[a] The results were averages of triplicates. Aminopolystyrene beads were heated at 100 °C for 4 h with 300 nmol maltose in 0.3 ml sodium phosphate buffer (0.1 M, pH 6.6).
[b] α-glucosidase, followed by glucose oxidase.

Morgan-Elson test. Analysis of *N*-acetylglucosamine was carried out by a modification of the method of Strominger *et al.* [13]. The stock Morgan-Elson reagent was prepared by dissolving dimethylaminobenzaldehyde (16 g) in glacial acetic acid (75 ml), adding concentrated hydrochloric acid (5 ml) and diluting to 100 ml with acetic acid. The stock reagent was diluted (2:5) with acetic acid before use. The beads were heated in *N*-acetylglucosamine solutions in water. To aliquots (3 × 75 µl) of supernatant, disodium tetraborate solution in water (5%, 75 µl) was added and the tubes heated for 7 min at 100 °C, followed by immediate cooling in an ice bath. Aliquots (700 µl) of diluted Morgan-Elson reagent were added to each tube and the mixed solutions were heated at 37 °C for 20 min and read immediately at 585 nm. The sugar remaining in the supernatants was estimated by comparison with standard *N*-acetylglucosamine solutions.

(*d*) *Enzymatic analysis of bound and unbound maltose*

Binding of maltose to beads, enzymatic hydrolysis and glucose analysis were all carried out in sodium phosphate buffer (0.1 M, pH 6.6). The binding supernatants were used for both enzymatic analysis [14] and a phenol-sulfuric acid measurement (Table 1).

Enzymatic hydrolysis of maltose. Treatments with α-glucosidase were carried out in glass tubes using the following samples: (1) replicates of one maltose-substituted bead, immersed in buffer (100 µl); (2) aliquots (100 µl) of the binding supernatant; and (3) maltose standard solutions in buffer (200 µM, 100 µl). The samples were incubated with α-glucosidase solution (1 U ml^{-1}, 10 µl) with shaking at 37 °C for 1.5 h.

Enzymatic analysis of glucose. A stock enzyme solution contained glucose oxidase (0.9 U ml^{-1}) and horseradish peroxidase (0.4 U ml^{-1}) in sodium phosphate buffer. The test reagent was prepared immediately before use by mixing enzyme solution (6 ml) with a solution of ABTS substrate (0.115 mM, 12 ml). The solutions from the hydrolysis step were tested directly, together with a series of glucose standards containing 1–9 µg glucose in buffer (100 µl).

Table 2. Immunochemical analysis of polymer-bound maltose.[a]

Bound sugar	*Absorbance (412 nm)*	*Maltose/lactose ratio*
Maltose	0.948	13.2
Lactose	0.072	
Maltose	1.044	8.2
Lactose	0.128	
Maltose	1.288	7.0
Lactose	0.183	
Maltose	1.154	8.3
Lactose	0.139	

[a] The results were averages of quadruplicates, obtained using four different batches of aminopolystyrene beads, heated at 100 °C for 3 h with 0.1 mM sugars in sodium phosphate buffer (0.1 M pH 5.6).

Aliquots (750 µl) of test reagent were added to each tube, then incubated at 37 °C for 20 min and read at 420 nm.

(*e*) *Immunochemical analysis of bound maltose*

Phosphate-buffered saline (PBS-T) was 0.14 M NaCl containing sodium phosphate buffer (0.01 M pH 7.4) and 0.05% Tween-20. Beads were incubated with 0.1 mM maltose or lactose solutions in sodium phosphate buffer (pH 4.3) at 100 °C for 3 h.

The beads were blocked for 2 h with either 1% gelatin or 10% Boehringer Mannheim Blocking Reagent, followed by four washes with PBS-T, and incubated for 2 h with a 1:50 dilution of Concanavalin A in PBS-T containing 0.1 mM Mn^{2+} and Ca^{2+}. The beads were again washed four times with PBS-T and incubated for 2 h with a 1:20 dilution of rabbit anti-Concanavalin A, washed four times with PBS-T and incubated for 2 h with a 1:1000 dilution of sheep anti-rabbit IgG-horseradish peroxidase conjugate, washed four times with PBS-T and the beads transferred to a microtitre plate, with one bead per well. A solution of mannose (0.5 M in PBS-T, 100 µl) was added to each well and incubated at 25°C for 15 min. The beads were then removed and ABTS substrate solution (100 µl) added [15]. The mixture was incubated with shaking and the colour development stopped by the addition of sodium azide (1 mM, 50 µl) after an appropriate time (30–60 min). The results were expressed as ratios of absorbances obtained from the maltose-substituted beads to those from the lactose-substituted beads as a negative control (Table 2).

Results

Derivatization of polystyrene beads

Nitration was carried out at a low temperature to minimize physical damage to the beads. If the temperature of the reaction mixture was allowed to rise to 20 °C, the beads adhered in clumps, resulting in uneven nitration of the

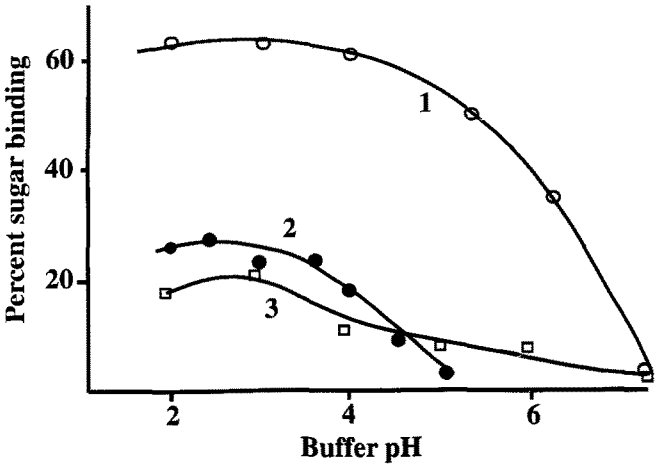

Figure 2. Binding of reducing sugars to aminopolystyrene at 100 °C as a function of pH in 0.1 M sodium citrate-0.2 M sodium phosphate buffers. Curve 1: 1 mM maltose for 2 h, analysed by the phenol-sulfuric acid test; curve 2: ^{14}C-glucose for 0.5 h; curve 3: ^{14}C-*N*-acetylglucosamine for 2 h.

surface. Consistent results were obtained with a reaction for 3 h at 5 °C. Longer times resulted in more deeply coloured beads, because of a higher degree of nitration; if the temperature was maintained below 3 °C, the rate of nitration was quite slow.

Reduction with stannous chloride was carried out at between 75 °C and 90 °C for 2–3 h. Higher temperatures gave more efficient reduction but, above 90 °C, the beads became soft and sticky, although they maintained acceptable sugar-binding properties. The degree of derivatization was typically 500–700 nmol of amino groups per bead after reduction at 90 °C. Milder chemical reductions, such as with dithionite or titanium(III) chloride, were also explored, but the degree of reduction was much lower. The reduced beads were stored at 4 °C under 1 M HCl to prevent oxidation of the amino groups. The sugar-binding characteristics of the beads were stable over several months.

BINDING OF SUGARS TO DERIVATIZED POLYSTYRENE BEADS

Monosaccharide analysis

The binding of ^{14}C-glucose and ^{14}C-*N*-acetylglucosamine to aminopolystyrene beads was determined by heating the beads with solutions of the labelled sugars and measuring the activity left in the supernatants. The influence of the buffer pH on the rate of binding (Fig. 2) was determined for short heating times, to approximate initial-rate conditions. The extent of binding of the sugars was also investigated for different heating times (Fig. 1).

The stability of binding of ^{14}C-glucose and ^{14}C-*N*-acetylglucosamine to beads, after heating at 100 °C for 2 h in buffers from pH 2.0 to 7.0, was investigated by allowing them to stand first in PBS-T for 16 h, to assess whether the sugar binding was adequate for immunochemical experiments, and then in 3 M acetic acid for 4 h to release any glycosylamine present [17]. At no stage was significant radioactivity observed in the supernatants.

The extent of binding of solutions of maltose and *N*-acetylglucosamine to aminopolystyrene beads under various conditions was determined by analysis of the supernatants by the phenol-sulfuric acid [12] and Morgan-Elson [13] tests, respectively. The extent of binding (Fig. 1) was consistent with those obtained by radiochemical analysis.

Maltose analysis

The kinetics of binding maltose were similar to those for glucose and, under optimal conditions, binding was essentially complete (Fig. 1). The accessibility of the bound maltose was determined by an assay of glucose released from the beads by α-glucosidase. The results (Table 1) show that 43–52% of the bound maltose was accessible to hydrolysis by the enzyme.

The binding of maltose was also assessed by an enzyme-linked lectin-binding assay. The lectin, Concanavalin A (Con A), which has a specificity for α-D-mannopyranosides > α-*N*-acetyl-D-glucosaminides, was used to demonstrate the presence of maltose on the beads, using bound lactose as a control.

Non-specific binding of proteins to the polystyrene matrix proved troublesome, and was aggravated by the introduction of the amino groups. Modification of the amino groups, by acetylation or coupling with erythrose, had no effect. Several methods of protein blocking, such as 2% bovine serum albumin and 1% gelatin, were used, but 10% Boehringer Mannheim Blocking Reagent proved the most effective. Alternatively, high background readings were obviated by means of hapten displacement of the Concanavalin A–Antibody complex, using methyl α-D-mannopyranoside or mannose (Table 2).

Discussion

The purpose of the present study was to evaluate simple aqueous chemistry for the covalent immobilization of reducing sugars to polymer supports. For effective attachment of a ligand to a polymer support, the coupling reaction must be rapid, it must be thermodynamically favourable and the reaction must proceed under acceptably mild conditions. Moreover, the immobilized ligand must be held in a stable linkage and be sterically accessible for chemical and biochemical reactions. To date, methods for the attachment of reducing sugars to supports have required high concentrations of sugar and long reaction times [5]. The results of this and another recent study [11] demonstrate that it is possible to achieve good attachment of sugars to polymer supports using simple glycosylamine chemistry.

When reducing sugars were heated with polystyrene

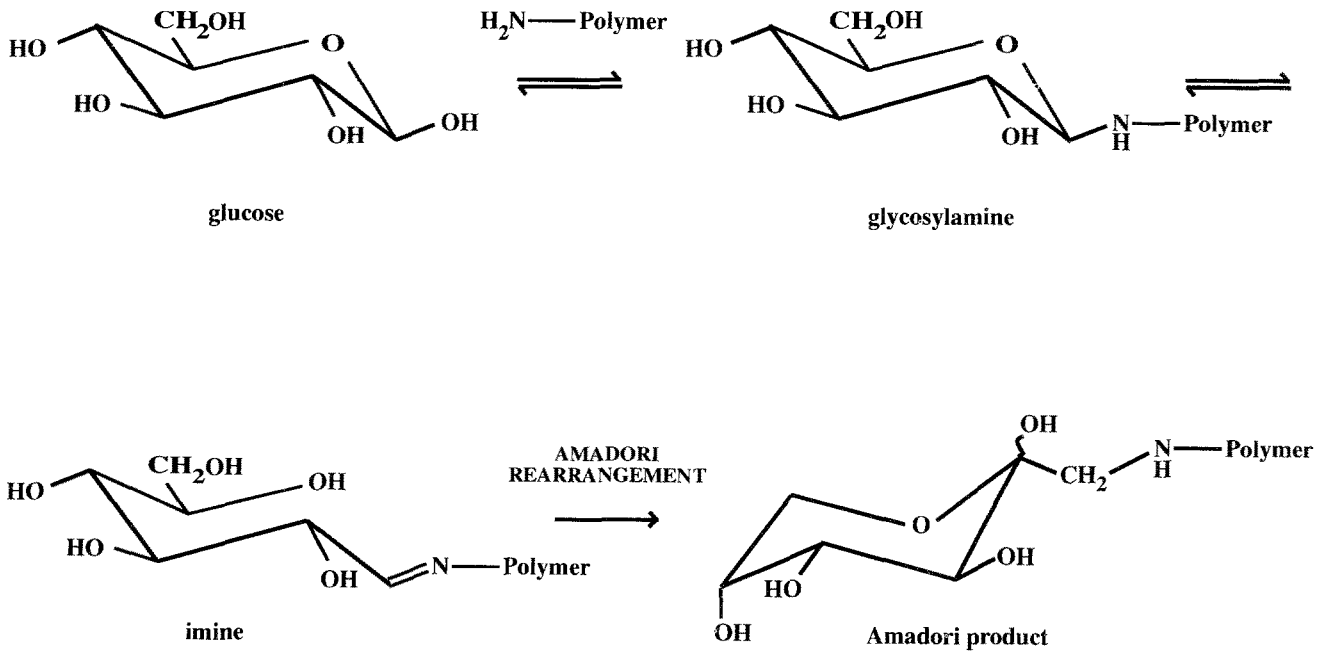

Figure 3. Proposed steps in the reversible formation of an aminopolystyrene-bound glycosylamine of glucose and its irreversible conversion to an Amadori product.

substituted with primary amino groups, rapid binding was observed. It was most rapid at mildly acidic pH values (Fig. 2) and conformed to the expected first-order kinetics, with an approximately constant half-life over the range 10^{-7}–10^{-4} M, so that the proportion of sugar bound in a particular time is independent of concentration, and the method can be used for very small amounts of sugar. The experiments with radiolabelled glucose and *N*-acetylglucosamine used sugar concentrations of about 100 nM and 500 nM, respectively, corresponding to 10 and 50 pmol per bead. The rate of binding is expected to be proportional to the concentrations of amino groups on the polymer, and the half-life for sugar binding can be decreased by increasing the level of functionalization of the beads.

Glycosylamines are known to be rather labile to hydrolysis [16] but the binding of sugars to aminopolystyrene was irreversible under competition conditions (acidified benzaldehyde), in 3 M acetic acid (which is expected to hydrolyse glycosylamines [17]) and on extended incubation in buffer at neutral pH. The binding of the sugars is most effective under mildly acidic conditions (Fig. 2), which are known to favour the Amadori rearrangement [18]. It is likely that the sugars undergo a rapid and reversible formation of glycosylamines, which are converted irreversibly, through the open-chain amino form and a slower Amadori rearrangement (Fig. 3), to 1-aminodeoxyketoses, which are not labile to hydrolysis.

Previous work [11] involved the binding of several oligosaccharides, in methanolic solutions, to immobilized primary aliphatic amines in the base form. An Amadori rearrangement does not appear to be involved. Instead, a glycosylamine is formed under equilibrium control and then stabilized by acetylation to a glycosylamide. Sugars with reducing-terminal *N*-acetylglucosamine residues were bound with relatively low efficiency.

The present study shows that useful binding of *N*-acetylglucosamine can be obtained at mildly acidic pH (Fig. 2) and that the binding is stable without *N*-acetylation. The rate of binding of *N*-acetylglucosamine is about one-third that of glucose (Fig. 1) and strongly pH-dependent. Glycosylamines of 2-acetamido sugars are not known to undergo the Amadori rearrangement, but the stability of the binding indicates that some such process must take place, albeit at a slower rate than the Amadori rearrangement of aldoses. The requirement for heating at an acidic pH may restrict the method to acid-stable sugars. If so, it could not be used for sialylated glycans of either *N*- or *O*-linked glycoproteins, but acceptable bindings were obtained at pH values as high as 6.6 (compare Tables 1 and 2).

Our prototype binding system does not provide spacer arms for the sugar-binding sites, and locates the sugars quite close to a highly hydrophobic surface. Using Concanavalin A to probe polymer-bound maltose, however, it is clear that a significant population of the maltose residues is still sterically accessible to the immunoassay reagents. Moreover, a significant proportion is also accessible to enzymic modification, such as by α-glycosidase.

We envisage that one application of immobilized sugars will be to exploit the obvious advantages of a solid-phase system to carry out sequential exoglycosidase treatments and immunochemical probing with lectins and antibodies

[19, 20]. Since it is possible to bind picomolar amounts of sugars, with either aldose or 2-acetamidoaldose reducing terminal residues, the approach has considerable potential for exploratory analysis of oligosaccharides, for evaluation of lectins and antisera, and as a complement to chromatographic methods. The present study confirms and extends a recently reported method [11] for sugar immobilization. In particular, it demonstrates that, given favourable thermodynamics, simple aqueous chemistry can be used to bind reducing sugars.

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