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Binding of carbohydrates to solid supports part 2: reaction of sugar hydrazones with isothiocyanate-substituted polystyrene

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The binding of sugars to a polymer support as thiosemicarbazones has been investigated as a means of immobilizing glycans. Hydrazones of glucose and *N*-acetylglucosamine were prepared by reaction with hydrazine hydrate, and successfully reacted with isothiocyanate-substituted polystyrene by incubation at room temperature and neutral pH. The binding was efficient and stable in aqueous buffers over a range of pH conditions. The bound sugars were recovered in moderate yield by treatment of the beads with hydrazine hydrate, benzaldehyde or acetone. Direct binding of reducing sugars to thiosemicarbazide-substituted polystyrene was not successful because of the unfavourable thermodynamics.

Keywords: sugar hydrazones; immobilization; aminopolystyrene; isothiocyanatopolystyrene; thiosemicarbazone.

Introduction

In view of the developing interest in the importance of the glycosylation of proteins [1–3], it has become essential to develop new methods for the study of the detailed structure of complex glycans. We have undertaken [4] a study of methods for the immobilization of glycans on solid supports which can be applied to affinity chromatography [5], immunoassay [6–8] and antibody production [9].

To date, the most common method for immobilizing reducing sugars has been reductive amination [10], whereby the sugars are first attached to polymer-bound primary amino groups by a labile glycosylamine linkage which is then stabilized by reduction. This reduction is slow and the efficiency of binding is rather low when limited amounts of sugars are available [10]. In recent variations on the approach, the glycosylamine linkage is stabilized by acylation [5] or Amadori rearrangement [4].

We now report an improved procedure which achieves efficient immobilization of reducing sugars as thiosemicarbazones. It has the advantages of using mild coupling conditions, suitable for glycans containing acid-labile linkages, and permitting subsequent removal of the sugars. This combination of properties and the chemical inertness of the polystyrene support make the procedure potentially suitable for use in solid-phase sequencing and synthesis of sugars.

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Experimental

Polystyrene beads with specular finish (3.2 mm in diameter) were from the Precision Plastic Ball Co. (USA) and alkylamine beads (6.3 mm diameter, 3 mmol amine function per bead) from Pierce Chemical Co. (USA). D-[U-^{14}C] Glucose (3 mCi mmol^{-1} in 3% ethanol) and *N*-acetyl-D-[1-^{14}C]glucosamine (57 mCi mmol^{-1} in 2% ethanol) were from Amersham International plc (England), hydrazine hydrate and thiophosgene from Aldrich Chemical Co. (USA) and Ultima Gold scintillant from Packard (Holland). All other chemicals were of analytical grade. Buffers of sodium phosphate (0.2 M) and sodium citrate-phosphate (0.1 M: 0.2 M) contained 0.002% chlorhexidine as preservative. Phosphate-buffered saline (pH 7.4) contained 0.01 M sodium phosphate, 0.14 M NaCl and 0.05% Tween-20. NMR studies were carried out on a Varian XL-400 spectrometer in D_2O at 25 °C. Proton chemical shifts were measured relative to internal trimethylsilylpropanesulfonic acid.

Kinetics of formation of sugar hydrazones in hydrazine hydrate

Glucose or *N*-acetylglucosamine (10 mg) was dissolved in 100% or 25% hydrazine hydrate (400 µl) and allowed to stand at room temperature for up to 6 h. The reagent was removed in a stream of dry nitrogen and the product dissolved in deuterium oxide for ^{1}H NMR spectroscopy. The degree of conversion to the hydrazones and loss of *N*-acetyl groups were determined by comparison of the

Table 1. NMR reporter resonances for analysis of sugar derivatives in deuterium oxide.

Compound	*Resonance*	*Assignment*
Glucose	5.21 (d, J = 3.8)	H-1 α-pyranoside
	4.62 (d, J = 7.9)	H-1 β-pyranoside
N-acetylglucosamine	5.19 (d, J = 3.4)	H-1 α-pyranoside
	4.70 (d, J = 8.4)	H-1 β-pyranoside
Glucose hydrazone	4.06 (d, J = 9.0)	H-1 β-pyranoside
	7.27 (d, J = 6.5)	H-1 open-chain E-isomer
	6.70 (d, J = 6.5)	H-1 open-chain Z-isomer
N-acetylglucosamine hydrazone	2.03 and 2.04 (s, 3H)	*N*-acetyl
	4.08 (d, J = 9.6)	H-1 β-pyranoside
	7.24 (d, J = 5.3)	H-1 open-chain E-isomer
	6.55 (d, J = 7.4)	H-1 open-chain Z-isomer
Acetohydrazide	1.94 (s, 3H)	*N*-acetyl
4-tolylthiosemicarbazide	2.33 (s, 3H)	Methyl
	7.26 (s, 4H)	Aromatics
Glucose 4-tolylthiosemicarbazone	2.35 (s, 3H)	Methyl
	4.17 (d, 1H, J = 8.8)	H-1 β-pyranoside
	7.26 (s, 4H)	Aromatics

resonances (Table 1) corresponding to H-1 and the *N*-acetyl groups of *N*-acetylglucosamine, *N*-acetylglucosamine hydrazone and acetohydrazide.

Preparation of labelled sugar hydrazones

A solution of [^{14}C]glucose (approx. 20 nmol) in 70% aqueous ethanol (10 µl) was evaporated to dryness in a 1.5 ml plastic centrifuge tube. The residue was dissolved in hydrazine hydrate (25 µl) and allowed to stand at room temperature for 30 min before evaporation in a vacuum desiccator containing concentrated sulfuric acid. The evaporation was complete after 2 h. To ensure effective removal of hydrazine, the residue was twice dissolved in water (50 µl) and reevaporated. *N*-acetyl [^{14}C]glucosamine was treated in the same way with 25% aqueous hydrazine hydrate for 6 h before evaporation. The sugar hydrazones were stored dry in the freezer for up to 2 months.

Preparation of 4-tolylthiosemicarbazide

Tolyl isothiocyanate was prepared from 4-toluidine (10.7 g, 0.1 mol) as described [11] and dissolved in chloroform (75 ml). Hydrazine hydrate (50 ml, 1 mol) was added, followed by ethanol (50 ml) to form a homogeneous solution and the solution allowed to stand for 30 min. The white crystals were collected by filtration and crystallized from ethanol. The yield was 9.1 g (50%), melting point 133–134 °C.

Preparation of glucose 4-tolylthiosemicarbazone

Glucose (1 g, 5.56 mmol) and 4-tolythiosemicarbazide (1 g, 5.52 mmol) were mixed with ethanol: water (1:1, 40 ml) and acetic acid (0.50 ml) to form a cloudy solution [12], which was refluxed for 1 h and left standing overnight at room temperature. A small amount of insoluble material was removed by filtration and the filtrate evaporated to give a foam (2.1 g), which was purified by flash chromatography on a silicic acid column. Unreacted 4-tolylthiosemicarbazide eluted with ethyl acetate and glucose 4-tolylthiosemicarbazone with ethanol:ethyl acetate (1:9). The yield was 0.80 g (40%).

Stability of glucose 4-tolylthiosemicarbazone

A solution of glucose 4-tolylthiosemicarbazone (10 mg, 30 mmol) in D_2O (0.5 ml) was kept at 27 °C and its ^{1}H-NMR spectrum measured at intervals over a period of 14 days. The solution was subsequently heated at 80° for 4 h and the spectrum again measured. The absence of glucose was judged by the lack of H-1 resonances (Table 1). NMR studies of the stability of *N*-acetylglucosamine 4-tolylthiosemicarbazone in aqueous solution were not carried out because of its poor solubility in water.

Kinetics of formation of glucose 4-tolylthiosemicarbazone

Glucose (1.53 mg, 8.5 µmol) and 4-tolylthiosemicarbazone (1.54 mg, 8.5 µmol) were dissolved in a 50 mM solution of potassium dihydrogen phosphate in D_2O (0.5 ml, apparent pH 4.6) and maintained in an NMR tube at 80° for 16 h. ^{1}H-NMR spectra were measured at intervals and the reporter groups (Table 1) for glucose, 4-tolylthiosemicarbazide and glucose 4-tolylthiosemicarbazone used to determine the course of the reaction.

Derivatization of polystyrene beads

Aminopolystyrene beads were prepared as before [4] in batches of 500 beads and stored in 0.1 M hydrochloric acid at 4 °C. They typically had 750–800 nmol amino groups per bead, as assessed by reaction with 4-nitrobenzaldehyde [4].

Isothiocyanatopolystyrene beads were obtained by washing batches of 50 aminopolystyrene beads with water and ethanol, adding them to a solution of thiophosgene (20 µl, 0.26 mmol) in ethanol (1.25 ml) and agitating gently for 2 h. They were then collected by filtration and washed several times with ethanol to remove all traces of excess reagent. The level of residual amino groups was determined by the 4-nitrobenzaldehyde method [4] and the difference between this level and that of the initial amino beads was taken to be the level of conversion to isothiocyanato groups. Commercial alkylamino beads were treated in the same way.

Thiosemicarbazido beads were prepared in batches from 50 isothiocyanatopolystyrene beads by washing with ethanol and agitating gently in a solution of hydrazine hydrate in ethanol (5%, 5 ml) for 30 min. The beads were collected by filtration, washed with ethanol and stored in 0.01 M hydrochloric acid at 4 °C.

Binding of sugar hydrazones to isothiocyanatopolystyrene beads

Before binding, the isothiocyanatopolystyrene beads were equilibrated with phosphate buffer (0.2 M) of the same pH as that used for subsequent binding for 15 min and drained. In a typical experiment, duplicate sets of three beads were added to a solution of [^{14}C]glucose hydrazone (2 nmol) or *N*-acetyl [^{14}C]glucosamine hydrazone (0.1 nmol) in buffer (200 µl) and agitated gently for the requisite time. Aliquots (2 × 50 µl) of the supernatants were taken for radiochemical counting. Binding was evaluated in buffers of different pH for 1 h and a detailed time course determined at pH 8.0.

Removal and recovery of bound sugars from isothiocyanatopolystyrene beads

To establish the stability of binding of sugars at different pH values, three beads were added to 200 µl of specified buffer and agitated gently for 2 h. All experiments were performed in duplicate in sealed tubes and aliquots (2 × 50 µl) of supernatant were taken for radiochemical counting. To determine the efficiency of recovery of the sugars, the beads were exposed in the same way to 200 µl of 100% or 25% hydrazine hydrate, and aliquots of supernatants counted as before. More forcing conditions employed hydrazine hydrate, ethanolic benzaldehyde and aqueous acetone at higher temperatures.

Binding to alkyl isothiocyanato beads

Before binding, the alkyl isothiocyanato beads were equilibrated with phosphate buffer (0.2 M) for 15 min and drained. One bead was added to a tube containing a solution of *N*-acetyl [^{14}C]glucosamine hydrazone (0.5 nmol) in the same buffer (250 µl) and agitated gently for 1 h. Duplicate experiments were carried out and aliquots (2 × 50 µl) were taken for radiochemical counting.

Results

Hydrolysis of glucose 4-tolylthiosemicarbazone

The ^{1}H-NMR spectrum of an aqueous solution of glucose 4-tolylthiosemicarbazone did not change after 14 days at 27 °C and subsequent heating at 80 °C for 4 h, indicating that no hydrolysis had taken place.

Kinetics of formation of glucose 4-tolylthiosemicarbazone

The kinetic study of the reaction between glucose and 4-tolylthiosemicarbazide in deuterium oxide produced a straight-line plot using the linear form of the rate equation expected for an $A + B = Z$ reaction approaching equilibrium. The equilibrium constant for the formation of the product was 38 M^{-1}. Assuming that the effective concentration of immobilized thiosemicarbazido groups is equivalent to that in a homogeneous solution containing the same total number of groups, a loading of 0.6 mmol of groups per bead would lead to 34% binding of glucose using three beads and 200 µl of buffer.

Derivatization of polystyrene beads

Polystyrene beads were nitrated and reduced as before [4]. Ethanol was a satisfactory solvent for the conversion to the isothiocyanate. Dioxan and tetrahydrofuran were evaluated, and they caused unacceptable softening of the beads.

When the amino content of beads was determined using 4-nitrobenzaldehyde [4], before and after treatment with thiophosgene, about 25% of the amino groups were still present after the reaction. The decrease in the level of amino groups was taken as a measure of the isothiocyanato loading (approximately 600 nmol per bead).

Binding of reducing sugars to thiosemicarbazido beads

The binding of glucose and *N*-acetylglucosamine to thiosemicarbazido beads was evaluated after heating under conditions similar to those used with amino beads [4], but the efficiency of uptake (Table 2) was poor. Moreover, only a low proportion of the bound sugars was removed by hydrazine, suggesting that the binding that had occurred was due mainly to Amadori attachment to the amino groups still present on the beads.

Binding of sugar hydrazones to isothiocyanato beads

Glucose and *N*-acetylglucosamine were treated in 100% hydrazine hydrate at room temperature for various times, then evaporated under reduced pressure. The conversion to the hydrazones was complete after treatment for 30 min,

Table 2. Direct binding[a] of [^{14}C]glucose to thiosemicarbazido- and aminopolystyrene beads and its recovery[b] with hydrazine hydrate.

Thiosemicarbazidopolystyrene		*Aminopolystyrene*	
bound (%)	*recovery (%)*	*bound (%)*	*recovery (%)*
48	6	90	1
46	4	91	0

[a] 0.2 M sodium phosphate buffer pH 3, 100°, 2 h.
[b] 100% hydrazine hydrate, room temperature, 16 h.

Table 3. Binding[a] of sugar hydrazones to isothiocyanate-substituted beads as a function of pH.

pH	*Glucose hydrazone (%)*	N-*acetylglucosamine hydrazone (%)*[b]	N-*acetylglucosamine hydrazone (%)*[c]
4	ND[d]	ND[d]	18
5	12	12	14
6	23	27	6
7	30	34	8
8	29	36	5
9	29	38	7

[a] 0.2 M sodium phosphate buffer, room temperature, 1 h.
[b] Isothiocyanatopolystyrene beads.
[c] Isothiocyanatoalkyl beads.
[d] Not determined.

but 21% of the N-acetyl groups were lost from the *N*-acetylglucosamine hydrazone. In 25% hydrazine hydrate, conversion to the hydrazones required treatment for 6 h, but the loss of *N*-acetyl groups was only 10%. As a general procedure, therefore, labelled *N*-acetylglucosamine was treated with 25% hydrazine hydrate for 6 h. The unpurified products were used to bind to isothiocyanato beads and no attempt was made to determine the yield of the hydrazones, but the high overall efficiency of immobilization of the sugars (Tables 3, 4) was consistent with a good conversion.

Binding of glucose and *N*-acetylglucosamine hydrazones at room temperature was optimal at a pH of 7–9 (Table 3). Time course experiments (Table 4) at pH 8 showed 76% binding of glucose hydrazone and 83% binding of *N*-acetylglucosamine hydrazone after 24 h.

The binding to alkylisothiocyanato beads (Table 3) was inferior to that of the aryl isothiocyanato beads and was most efficient at low pH.

Stability of binding and recovery of bound sugars

The attachment of the sugars was stable in buffers of pH 5–7.4 (Table 5). When the beads were treated with hydrazine hydrate at room temperature, there was a modest recovery of the sugar hydrazones (Table 6), but heating (Table 7) removed most of the bound sugars. These results suggest that there is more than one population of bound sugars, and that only some of them are released under mild conditions. Alternatively, the immobilized sugar hydrazones were treated with a solution of benzaldehyde or acetone at 100 °C (Table 7). Similar levels of recovery of the sugars were obtained.

Table 4. Time course of binding of sugar hydrazones to isothiocyanatopolystyrene beads[a].

Time (h)	*Glucose hydrazone (%)*	N-*acetylglucosamine hydrazone (%)*
0.5	16	20
1	29	34
2	40	48
4	54	61
8	72	71
24	76	83

[a] 0.2 M sodium phosphate buffer pH 8.0, room temperature.

Table 5. Loss of sugars bound as thiosemicarbazones to isothiocyanatopolystyrene beads on standing in aqueous buffers[a].

Reagent	[^{14}C]*glucose (%)*	N-*acetyl* [^{14}C] *glucosamine (%)*
pH 2.2 citrate/phosphate	11	6
pH 3.0 citrate/phosphate	6	6
pH 4.0 citrate/phosphate	4	4
pH 5.0 phosphate	3	1
pH 6.0 phosphate	1	0.6
pH 7.0 phosphate	0.5	0.3
pH 7.4 phosphate-buffered saline	0.7	0.7

[a] At room temperature, 2 h.

Table 6. Recovery at room temperature of sugars bound as thiosemicarbazones to isothiocyanatopolystyrene beads.

Time (h)	*Glucose removed (%)*		N-*acetylglucosamine (%)*	
	25% hydrazine hydrate	*100% hydrazine hydrate*	*25% hydrazine hydrate*	*100% hydrazine hydrate*
1	28	55	19	35
2	36	50	27	46
4	40	57	33	45
8	50	57	37	40
16	52	59	43	47

Table 7. Recovery under forcing conditions of sugars bound as thiosemicarbazones to isothiocyanatopolystyrene beads.

Bound sugar	*Hydrazine hydrate*[a] *2 h*	*Hydrazine hydrate*[a] *24 h*	*Benzaldehyde*[b] *2 h*	*Acetone*[c] *2 h*
glucose	94	94	53	47
N-acetyl-glucosamine	81	96	67	45

[a] 100% hydrazine hydrate at 50 °C.
[b] 25% benzaldehyde in 95% ethanol at 100 °C.
[c] 25% acetone in water at 100 °C.

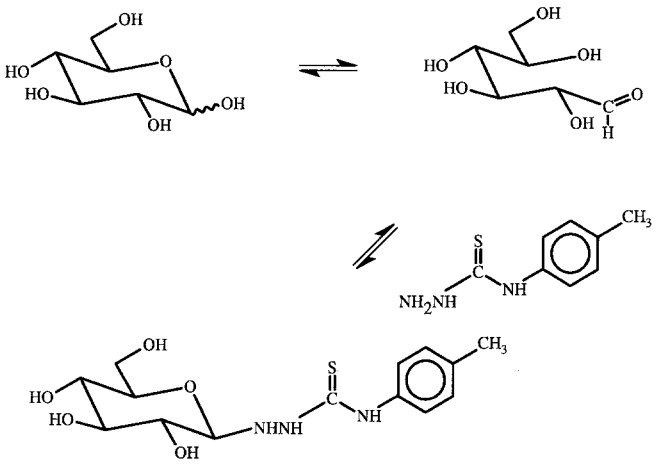

Figure 1. Direct formation of glucose 4-tolylthiosemicarbazone by condensation of glucose with 4-tolylthiosemicarbazide.

Discussion

We recently reported a method for the attachment of sugars to aromatic amino groups on polymers, using aminopolystryene as a prototype [4]. While the procedure was simple and the extent of binding quite high, it was necessary to employ protracted heating and mildly acidic conditions, which are inappropriate for glycans containing labile subunits, such as neuraminic acids. Moreover, the binding of the sugar involved an Amadori rearrangement and the formation of a permanent bond to the polymer, which limited the scope for recovery of the sugars. The present study was undertaken in the hope of devising a system which permits both linkage under mild conditions and the ready recovery of linked sugars, so that it can be used for solid-phase manipulations, such as the synthesis and structural modifications of sugars.

Thiosemicarbazones have been prepared in good yield by condensation of reducing sugars with thiosemicarbazides [12]. In general, aldose hydrazones exist in aqueous solution primarily in ring forms [13], especially the β-glycopyranosylhydrazines, but no information is available about their thermodynamic stability. The formation constant of a model compound, glucose 4-tolylthiosemicarbazone (Fig. 1), was determined by ^{1}H-NMR. Its low value ($38\ M^{-1}$) suggested that efficient binding of a sugar to thiosemicarbazide groups immobilized on a solid support would require relatively high concentrations of the functional groups on the support. Consistent with this, the direct binding of reducing sugars to thiosemicarbazido beads was poor (Table 2). Despite its modest thermodynamic stability, however, the good kinetic stability of glucose 4-tolylthiosemicarbazone on heating in unbuffered D_2O suggested that the thiosemicarbazone linkage might still be suitable for immobilization of sugars.

An alternative method of immobilization, employing reaction of preformed sugar hydrazones with isothiocyanato beads (Fig. 2) at room temperature, achieved good immobilization within a few hours (Table 4). Model experiments were undertaken to determine the conditions required for efficient hydrazone formation. Glucose and *N*-acetylglucosamine were treated with 25% and 100% hydrazine hydrate for up to 6 h and the products examined by ^{1}H-NMR spectrometry. Both sugars were converted quantitatively to their hydrazones after 30 min (in 100% hydrazine hydrate) or 6 h (in 25% hydrazine hydrate). There was concomitant loss of *N*-acetyl groups from *N*-acetylglucosamine, which amounted to some 21% or 10%, respectively. Both hydrazone formation and deacetylation have first-order kinetics with respect to the sugar and these observations can be extrapolated to the nanomolar amounts of labelled sugars used in binding experiments.

Optimal binding of hydrazones to isothiocyanatopolystyrene beads occurred in the pH range of 7–9 (Table 3), consistent with the requirement that the sugar hydrazones be deprotonated to enable nucleophilic attack on the isothiocyanates. The attached sugars were stable at room temperature under neutral conditions (Table 5), but hydrolysed slowly at pH 5 and below. Their stability under alkaline conditions was not tested.

An advantage of this method of coupling is that it is possible to release the sugars using nucleophilic conditions (Fig. 3). Up to 59% recoveries of [^{14}C]glucose, and 47% of *N*-acetyl-[^{14}C]glucosamine, as the hydrazones, were obtained by treating the beads with hydrazine hydrate at room temperature (Table 6). Almost complete recovery (Table 7) was obtained at 100 °C, but with the disadvantage that any *N*-acyl groups are lost. Cleavage under electrophilic conditions, by exchange on to an excess of benzaldehyde or acetone gave somewhat lower recoveries, but has the advantages that the free sugars are liberated (Fig. 3), rather than the hydrazones, and that there is no loss of *N*-acyl substituents.

Alkylaminopolystyrene beads are commercially available, and it was of interest to establish whether they are amenable to the binding of sugars by this isothiocyanate

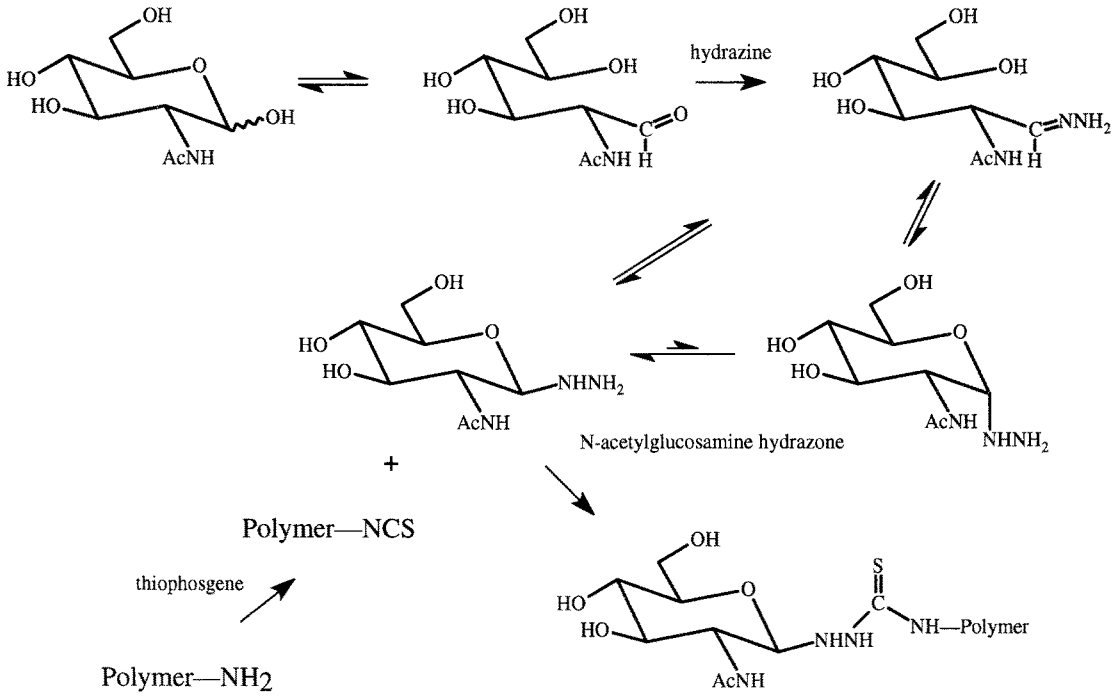

Figure 2. Formation of *N*-acetylglucosamine hydrazone and its reaction with an isothiocyanate-derivatized polymer to give the immobilized *N*-acetylglucosamine thiosemicarbazone.

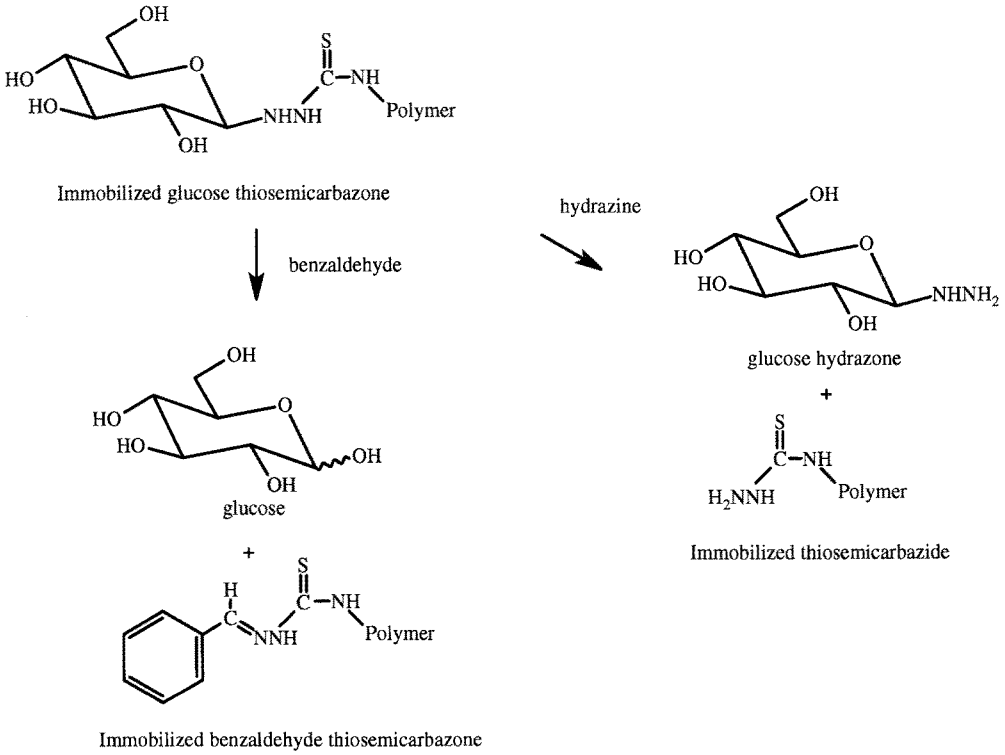

Figure 3. Liberation of immobilized glucose thiosemicarbazone by treatment with hydrazine to liberate glucose hydrazone and with benzaldehyde to form free glucose.

chemistry. In the event, the approach was only marginally successful (Table 3). Aliphatic isothiocyanate groups are less reactive to nucleophiles than their aromatic counterparts [14], which explains the more sluggish kinetics of coupling with the sugar hydrazones. The enhancement of coupling at low pH is indicative of acid catalysis, which may be exploited in some situations.

The present chemistry for the immobilization of sugars as aryl thiosemicarbazones is similar to that in use for the covalent attachment of proteins for *N*-terminal analysis [15], whereby immobilized isothiocyanato groups are reacted with nucleophilic ε-amino groups of lysine side chains. Isothiocyanate-substituted sugar derivatives have been employed [8, 16] in the attachment of sugars to protein amino groups. With this strategy, however, the reactive ligand must be purified before coupling to the polymer, which is less convenient than the present approach.

The attachment of preformed sugar hydrazones to immobilized isothiocyanato groups represents a useful advance over other methods, in that it provides experimental simplicity, good binding under mild conditions and a reasonable level of recoverability. It is compatible with the presence of acid-labile sugar linkages, and retains the desirable structural characteristics [17–20] of a reducing terminal sugar in the β-pyranose ring form and a spacer arm between the polymer support and ligand. It therefore promises to be suitable for a number of solid-phase chemical and biochemical techniques, such as methylation analysis, sequencing [3, 21] and structural modification [22].

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