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Glucose-silica, an improved medium for high-pressure gel filtration chromatography

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ABSTRACT

Glucose was covalently coupled to aminopropyl derivatized silica of various pore sizes. The reaction most likely involved Schiff's base formation between the aldehyde of glucose and the amine on the silica which was then reduced with $NaCNBH_3$. This glucose-silica behaved as a nearly ideal molecular sieving support for the high-pressure gel filtration chromatography of proteins and was superior in performance to many currently available supports.

INTRODUCTION

Gel filtration is size-exclusion chromatography that uses aqueous solvents and hydrophylic packings. Low-pressure gel filtration media such as dextrans, agaroses and polyacrylamides have been used both preparatively and analytically. High-pressure gel filtration chromatography (HPGFC)[1] should have enhanced resolution and reduced time requirements. The enhanced resolution necessarily means that much less quantity of sample would be needed for analytical purpose.

In recent years, HPGFC has been used extensively. Researchers have used the technique for quick isolation and analysis of proteins of interest. A rapid, sensitive protein assay employing HPGFC has recently been described[1]. More recently, Massom *et al.*[2] have described a HPGFC method useful for studying ligand binding by proteins. While the applications of HPGFC are potentially extensive and powerful, it is also clear that current media are less than ideal. Several of the current supports have pressure limitations well below the capabilities of modern chromatographs. To obtain supports capable of withstanding high pressures, macroporous silicas have commonly been used. To minimize the interactions of silica itself and its ionizable silanols with the sample, the silica surface is often chemically modified with some hydrophilic silanizing reagent such as the glycidiol-3-oxypropyl moiety. Even so, currently available columns, silica-based or non-silica-based, often show mixed-mode chromatographic behavior consistent with some interactions between the applied

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sample and either the support surface or the hydrophilic modifying group attached to it[2–9].

By coupling glucose to aminopropyl-silica, five hydroxyl groups are added to the silica surface. As a result, hydrophilicity is enhanced on the surface and mobile solutes are further protected from interacting with the silica. Columns prepared from glucose-silica were compared to many of the existing HPGFC columns and showed improved performance.

EXPERIMENTAL

Chromatography

The Chromatograph was a Gilson 9000 autoanalytical system outfitted with a Jasco UVDEC detector, a Tandy 3000HD computer, and the Gilson 714 software for data collection and analysis. Chromatography was at room temperature (20°C) throughout.

Preparation of glucose-silica

The starting material was 7-μm spherical 3-aminopropyl-silica of various porosities. When available, these were obtained from Alltech (Deerfield, IL, U.S.A.); otherwise, they were synthesized from 7-μm Macherey-Nagel (Düren, F.R.G.) silicas of the appropriate pore size as follows: 10 g silica were refluxed 4–6 h in 20% (w/v) 3-aminopropyltrimethoxysilane (Petrarch, Bristol, PA, U.S.A.) in toluene. The derivatized silica was then thoroughly washed with toluene, then methanol, and dried at 60°C overnight.

Glucose-silica was prepared by reacting 5 ml (1 mmol) of 0.2 *M* D-glucose, 10 m*M* sodium phosphate, pH 6.8, and 126 mg (2 mmol) $NaCNBH_3$ per gram of aminopropyl-silica. The mixture was constantly stirred at 60°C for 5 h. The reacted resin was then washed with water, acetone, and then dried at 60°C. The dried glucose-silica was then tested with Cd–ninhydrin[10] and was typically found to lack any detectable unreacted amines.

Initially, the disappearance of glucose from the reaction mixture was measured with the anthrone reagent[11] to follow the progress of the reaction. However, control reactions lacking in silica also showed a decrease in measurable glucose with time, albeit at a slower rate and to a lesser extent. Presumably, this was due to a slow reduction by $NaCNBH_3$ of glucose to sorbitol. While such controls allow the coupling reaction to be followed qualitatively, the method is cumbersome and an alternative procedure was developed.

In order to study the reaction rate more accurately, approximately 600 000 cpm of [3-^{3}H]D-glucose (New England Nuclear, Boston, MA, U.S.A.) was added to the 5 ml reaction mixture. At various times, 0.2-ml portions of the reaction mixture were removed and mixed with 0.8 ml of 0.1 *M* hydrochloric acid to immediately terminate the reaction. After a brief centrifugation, duplicate 0.4-ml portions of the supernatant were mixed with 5 ml of scintillation fluid and the amount of [^{3}H]glucose determined using a Packard Tri-Carb 4640 scintillation counter. Silicas, recovered from the centrifugation during the time course study were washed thoroughly and tested with the Cd–ninhydrin reagent.

Column testing

Glucose-silica supports of 60, 100, 300 and 500 Å pore sizes were made as described and packed into 100 × 4.6 mm I.D. columns. The mobile phase was 100 m*M* sodium sulfate, 20 m*M* sodium phosphate, pH 6.8 and the flow-rate was 1 ml/min throughout. For comparison, 100 × 4.6 mm I.D. columns of RoGel P (70 Å pore), SynchroPak GPC (60 Å), Macrosphere GPC (60 Å), and a 250 × 4.6 mm I.D. Macrosphere R (20 Å pore) column were obtained from Alltech and also tested under the same conditions. A 300 × 7.5 mm I.D. BioSil TSK-125 column (Bio-Rad Labs., Richmond, CA, U.S.A.) was also compared under the same conditions except that it was necessary to reduce the flow-rate to 0.7 ml/min to maintain a backpressure of less than 30 bar as suggested by the manufacturer.

Proteins with a wide range of molecular weights were used to test the columns. Low-molecular-weight tryptophan (mol.wt. 204) was used as a marker for total excluded column volume. The individual proteins were typically made up as 1 mg/ml stock solutions and various mixtures were then prepared. Each protein was also injected individually onto the various columns to confirm the identity of each peak in a mixture. The injection volume was 5 μl and detection was by absorption at 220 nm throughout.

A 300 × 6.2 mm I.D. column of 100 Å pore glucose-silica was also packed for comparison to the TSK column. For this comparison, the protein test mixture supplied by Bio-Rad with the TSK column was used.

To characterize the pH resistance of the various supports, the underivatized silica, aminopropyl-silica and glucose-silica from the same base silica and porosity were treated with different pH buffers for 2 h. After 2 h, the samples were centrifuged, the supernatants were removed, and the dissolved silica content was determined by the molybdate method[12] using a commercially available kit (Chemets, Calverton, VA, U.S.A.). To further investigate the pH stability of the glucose-silica, 100 ml of the pH 10 buffer (60 column volumes) was flowed at 1 ml/min through a 300-Å glucose-silica column (100 × 4.6 mm I.D.). Chromatograms obtained before and after the high pH treatment were compared.

RESULTS

The reaction used to prepare glucose-silica is depicted in Fig. 1. The chemistry used is mild and simple to carry out. The aldehyde moiety of D-glucose presumably reacts with the primary amine on aminopropyl-silica to produce a Schiff's base. The Schiff's base is then reduced by $NaCNBH_3$, a reductant which is relatively specific for Schiff's bases[13]. A temperature of 60°C was found to be optimal for this reaction.

Using [^{3}H]glucose as a radiotracer, it was found that 0.25 mmol of the D-glucose coupled per gram of the 300 Å pore aminopropyl-silica and 0.55 mmol/g for the 100-Å aminopropyl-silica. The reaction was found to follow (pseudo)first order kinetics and the half-time of the reaction was 26 min for the 300-Å and 28 min for the 100-Å resin with an average of 27 min overall (Fig. 2). Therefore, the 5-h reaction time used should allow the reaction to be about 99.9% complete.

The completion of the reaction was confirmed by the Cd–ninhydrin test. It was found that glucose-silica showed no color change in the Cd–ninhydrin reagent while the parent aminopropyl-silica appeared intensely orange-red in color following Cd–

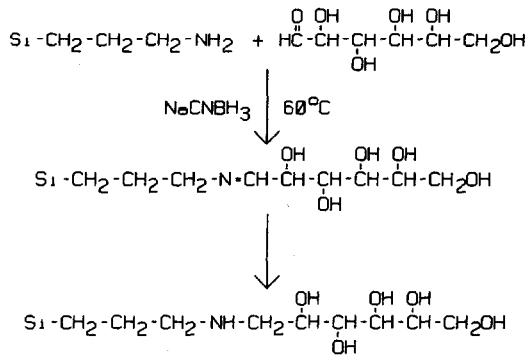

Fig. 1. A reaction overview. The amine of the aminopropyl-silica presumably forms a Schiff's base with the aldehyde of the D-glucose which is then reduced with sodium cyanoborohydride[13]. The two steps occur in a continuous fashion at 60°C in the presence of glucose and the reducing agent $NaCNBH_3$.

ninhydrin reaction. Cd–ninhydrin typically gives the same orange-red color with other primary amines (glycine, ethanolamine) tested. The addition of Cd to the ninhydrin reagent gives a more stable color than the typical purple color obtained with ninhydrin alone[10]. The progress of the reaction could also be following with Cd–ninhydrin during the time course shown in Fig. 2: silicas from short reaction time gave the characteristic orange-red color which progressively diminished until, as the glucose reaction reached completion, a pure white silica was observed.

The glucose-silica columns (60, 100, 300 and 500 Å) were tested with protein sizes ranging from immunoglobulin M (mol.wt. 1 000 000) to melittin (mol.wt. 2848). Fig. 3 shows the plots of molecular weight *vs.* retention time for the various pore sizes

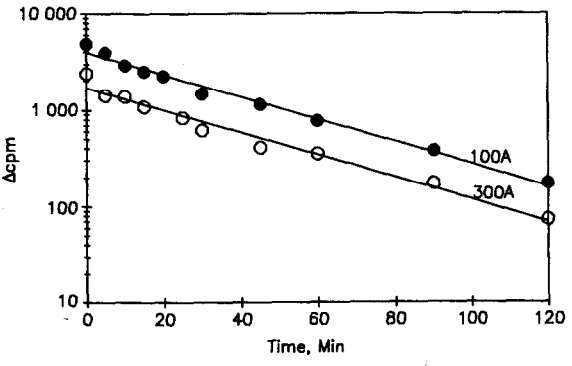

Fig. 2. Coupling of D-glucose to aminopropyl-silica follows (pseudo)first order kinetics. [^{3}H]Glucose was used to trace the coupling of glucose to two different pore size (100 Å and 300 Å) AP-silicas. The cpm remaining in the supernatant after reaction for a day was considered to represent the reaction endpoint and has been subtracted from the data (Δcpm). The semilogarithmic plot of Δcpm as a function of time was fit reasonably well by (pseudo)first order kinetics; the line shown was derived from the equation for a first order process. The average half-time of the reaction with either silica was 27 min. The difference in the vertical location of the curves is due to the greater quantity of the glucose coupled to the 100-Å resin as compared to the 300-Å resin which has a lower surface area and presumably, less aminopropyl groups to react.

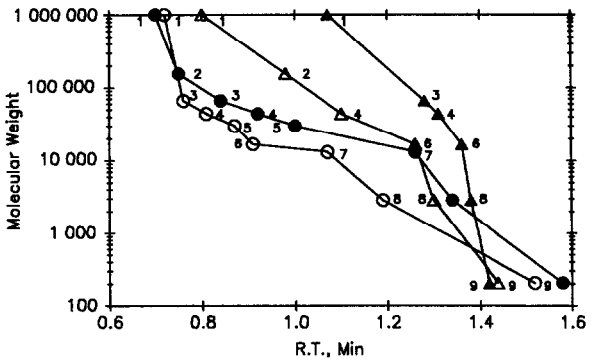

Fig. 3. Molecular weight *versus* retention time (R.T.) of various pore sizes. The proteins tested are indicated by the numbers. Proteins: 1 = immunoglobulin M (mol.wt. 1 000 000); 2 = immunoglobulin G (IgG) (156 000); 3 = bovine serum albumin (66 300); 4 = ovalbumin (44 000); 5 = carbonic anhydrase (30 000); 6 = bovine brain calmodulin (16 700); 7 = cytochrome *c* (13 000); 8 = bee venom melittin (2848). Number 9 is the amino acid tryptophan (204). Glucose-silica columns (100 × 4.6 mm I.D.) of different pore sizes are represented by open circles (60 Å), closed circles (100 Å), open triangles (300 Å) and closed triangles (500 Å).

and demonstrates the ability of glucose-silica to resolve proteins of different size classes. As expected, columns with larger pores were capable of resolving larger proteins.

As illustrated in Fig. 4, the performance of the 60-Å glucose-silica column was compared to four other commercial HPGFC columns: RoGel P, Macrosphere GPC 60, SynchroPak GPC 60 and Macrosphere GPC-R. When a mixture of three proteins

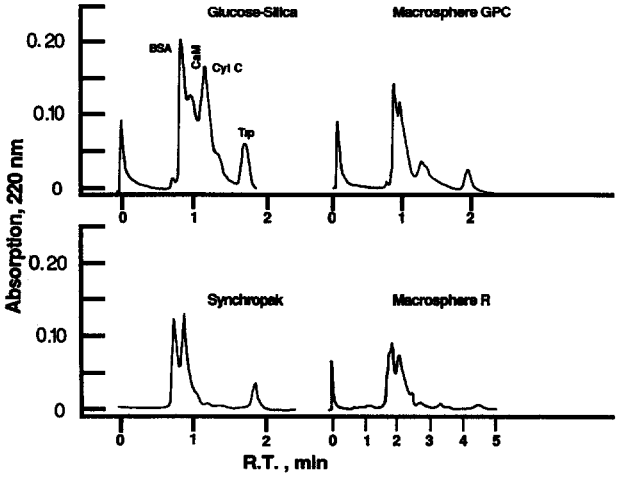

Fig. 4. A comparison of glucose-silica with other HPGFC columns. A mixture containing three proteins and Trp was injected onto four different columns: 60 Å pore glucose-silica, Macrosphere GPC 60 ("GPC"), SynchroPak GPC 60 and Macrosphere/R GPC ("R"). All columns were 100 × 4.6 mm I.D. except the last which was 250 × 4.6 mm I.D. All chromatograms are shown to the same scale and may be compared for protein recovery as well.

[bovine serum albumin (BSA), calmodulin (CaM) and cytochrome *c* (Cyt C)] and Trp was injected onto the columns, both the SynchroPak GPC 60 and the Macrosphere/R GPC could not resolve all four constituents in the mixture, yielding three and two peaks, respectively (Fig. 4). No peaks were observed at all with the RoGel column suggesting that this column bound all components of the mixture applied to it (data not shown). The glucose-silica column could not only separate all the constituents into four distinct peaks, it also gave the highest recovery of injected protein. The chromatograms shown (Fig. 4) are plotted to the same scale and represent the same amount of sample injected. The eye confirms the result obtained by integration that the glucose-silica column gave greater peak areas indicating that more of the applied protein elutes from the column.

Although the Macrosphere GPC 60 was able to resolve all the components in the above mixture, the chromatography of the individual proteins in the mixture demonstrated that retention time was not strictly dependent on protein size with this column. In fact, the Macrosphere GPC 60 support apparently has a strong repulsive interaction with the acidic protein CaM as shown in Fig. 5. As shown by this figure, calmodulin (mol. wt. 16 700) actually elutes before carbonic anhydrase (30 000) from the Macrosphere GPC column while they elute in the proper order from glucose-silica and at a retention time wholly consistent with each protein's molecular weight (see also Fig. 3).

A similar phenomenon was also observed with the TSK column. Again, calmo-

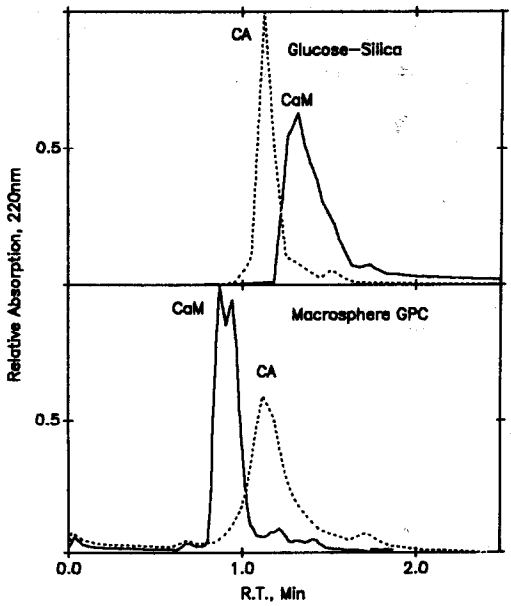

Fig. 5. Calmodulin behaves differently on glucose-silica and Macrosphere GPC 60. Samples of calmodulin (CaM) and carbonic anhydrase (CA) were injected onto the glucose-silica and the Macrosphere GPC 60 columns. CA, being a much larger protein than CaM, was observed to come off the glucose-silica column earlier than CaM, as expected. However, the Macrosphere GPC 60 demonstrated the reverse order of elution.

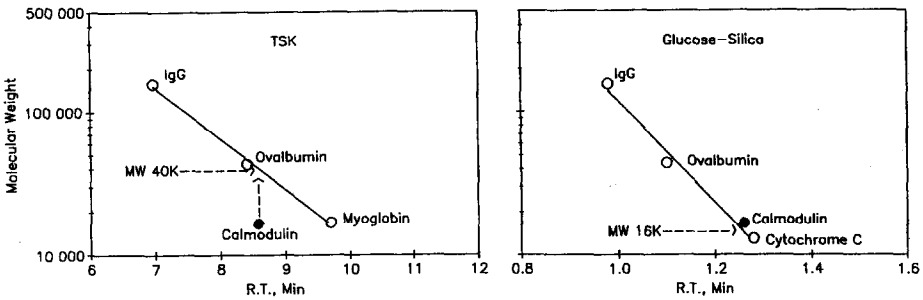

Fig. 6. Calmodulin's behavior on TSK is also unusual. CaM, a strongly acidic protein, behaved as though it were a 40 000 (40 K) molecular weight protein on the TSK column, while the glucose-silica gave a retention time for calmodulin more consistent with its true molecular weight. The apparent molecular weight calculated from the standard curve for calmodulin is indicated.

dulin was found to elute too soon for its small size. The acidic calmodulin behaved as though it were a 40 000 molecular weight protein on the TSK column (Fig. 6). In contrast, the glucose-silica column, once calibrated with the same standard proteins gave a molecular weight for calmodulin of 16 000, which is entirely consistent with its actual molar mass of 16 700 (ref. 14).

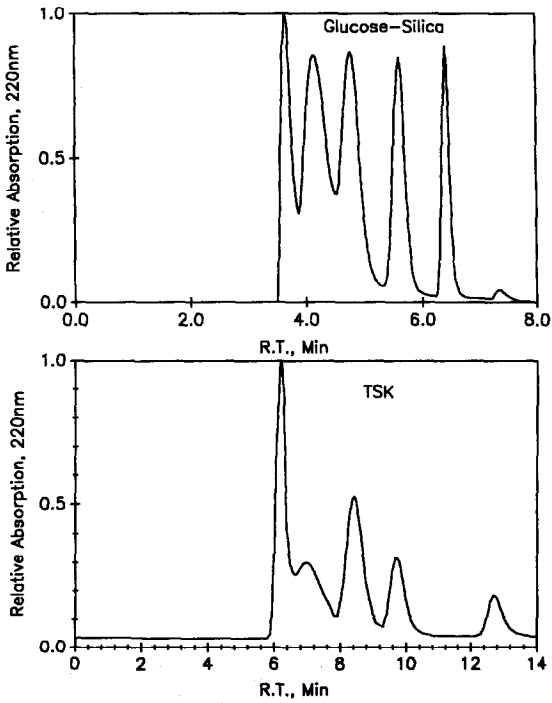

Fig. 7. Glucose-silica compared to TSK. The test mixture supplied by Bio-Rad with the TSK column was better resolved on glucose-silica. The test mixture contained, in order of elution, "protein aggregate", thyroglobulin (670 000), IgG, ovalbumin, myoglobulin (17 000), and vitamin B_{12} (1350).

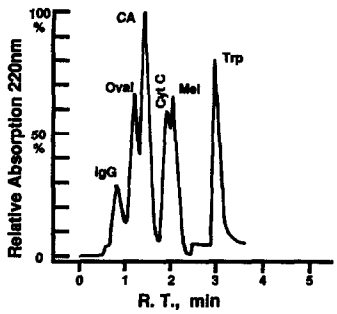

Fig. 8. Coupling two columns of different pore sizes extends the range of glucose-silica. Two 100 × 4.6 mm I.D. glucose-silica columns of different pore sizes (100 Å and 300 Å) were connected, outlet to inlet, with a short piece of tubing. A 10-μl mixture of five proteins and Trp was separated by the tandem-connected 100-Å and 300-Å columns connected together at the flow-rate of 1 ml/min. The last peak (Trp) appeared at approximately 3 min after the injections. Oval = Ovalbumin; Mel = melittin.

Furthermore, a 300 × 6.2 mm I.D. glucose column, though smaller than the TSK column 300 × 7.5 mm I.D., was capable of resolving all six components of the Bio-Rad protein standard test mixture while the TSK column could not (Fig. 7). The comparison also showed that the glucose column gave sharper peaks than the TSK column could. The glucose column clearly is superior to the TSK in resolving power as well as the ability to give correct molecular weights for acidic proteins like calmodulin.

To investigate whether a combination of pore sizes might extend the range of the glucose-silica column, a mixture of five proteins and Trp was injected onto two tandem-connected 100-Å and 300-Å glucose-silica columns. As shown in Fig. 8, all constituents were well resolved in 3 min at the flow-rate of 1 ml/min. This clearly demonstrated the powerful separation ability of glucose-silica as an HPGFC support.

Finally, glucose-silica was shown to possess strong resistance to high-pH solutions, as compared to the aminopropyl-silica and underivatized silica from which it

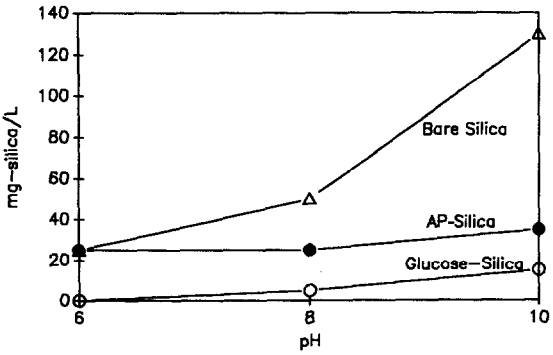

Fig. 9. Low pH sensitivity of the glucose-silica. Underivatized silica, 3-aminopropyl (AP)-silica and glucose-silica were treated with pH 6, 8 and 10 buffers (50 m*M* each in Tris, imidazole and boric acid titrated to the desired pH) for 2 h. The mixtures were then centrifuged and the supernatants removed for determination of dissolved silica. The glucose-silica resin showed strong pH resistance as compared to the 3-aminopropyl-silica and the bare silica.

was made (Fig. 9). For example, the pH 10 buffer dissolved only 0.01% of the glucose-silica in 2 h, as opposed to 0.13% for the bare silica. In a separate experiment, the chromatograms obtained before and after 100 ml of the pH 10 buffer (60 column volumes) flowed through the 300-Å column (100 × 4.6 mm I.D.) revealed no detectable difference in the resolution or peak area of the injected proteins (data not shown). The result suggested that the glucose on the silica surface may sterically block the pH sensitive silane linkage. A similar pH stability has been observed for a sterically blocked silica-based support in a reversed-phase column[15]. Thus, the pH stability of glucose-silica makes for a pH stable column. This could be an invaluable property if high pH mobile phases were necessary to an experiment.

DISCUSSION

A simple way of coupling glucose to macroporous silicas has been described. Fig. 1. represents only the presumed mechanism since the final product was only partially characterized chemically. The chromatographic behavior of the support is more germain and was the focus of the presented experiment. The resulting support proved to be superior to many commercially available HPGFC supports. The small 100 × 4.6 mm I.D. glucose-silica columns used for most of the studies here were sufficient to give good separation because of the high resolution of glucose-silica. A larger column is capable of even higher resolution (Figs. 7 and 8). All the tests performed have demonstrated that Glucose-Silica columns behave consistently with basic proteins (*e.g.*, melittin, lysozyme), acidic proteins (*e.g.*, calmodulin), and with large (immunoglobulin M) and small (melittin, cytochrome *c*) proteins. The glucose must nearly completely shielded the silica from interacting with the mobile phase and the solutes it contains as demonstrated by these results with various proteins and the demonstrated pH stability relative to silica. Glucose-silica appears to represent a significant improvement upon existing HPGFC supports.

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