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Short Communication

Chromatographic stability of glucose-silica

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ABSTRACT

Glucose-silica was prepared and packed into five 100 × 4.6 mm I.D. columns. These columns were tested initially with selected protein mixtures. Each of the columns was stored in one combination of buffer (100 m*M* sodium sulfate, 20 m*M* sodium phosphate, pH 6.8), temperature (4 or 20°C), methanol and sodium azide. After a year, we found that storing glucose-silica columns in the buffer containing sodium azide or methanol at 4°C was necessary to prolong stability in storage. However, glucose-silica also exhibited some dynamic instability.

INTRODUCTION

An important feature of modern high-performance liquid chromatography (HPLC) columns is greater stationary phase stability which increases their useful chromatographic lifetime. Alkaline buffer (*i.e.*, pH > 8) is known to reduce lifetime of columns packed with silica-based materials. Biodegradability is another problem associated with stability of HPLC packing materials [1].

Gel filtration is size-exclusion chromatography that uses mild aqueous solvents and neutral hydrophilic packings suitable for protein separations under non-denaturing conditions. We [2] and others [3,4] have reported the synthesis of glucose-bonded silica for such usage. In most cases, glucose was covalently coupled to aminopropyl-derivatized silica through Schiff's base formation and reduction with $NaCNBH_3$. Although the exact conditions used for synthesis varied among the researchers, we, in particular, concluded that glucose-silica was a well-behaved gel-filtration medium for HPLC of proteins. Later, after prolonged (about 6 months) column usage, we became aware that the performance of glucose-silica columns degrades with time. Our experience and that of others [4,5] suggested that we should further investigate this stability issue. Accordingly, this study addresses the static and dynamic stability of these columns. While the results obtained with glucose-silica stability only strictly apply to this chromatographic support, the results obtained may also suggest strategies for preserving the performance of other biocompatible columns.

EXPERIMENTAL

Chromatography

The chromatograph was a Rainin Rabbit-HP solvent-delivery system outfitted with a Knauer variable-wavelength monitor, a Macintosh SE

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computer, and the Rainin Dynamax version 1.2 software for data collection and analysis. Chromatography was at room temperature (20°C) with a 1 ml/min flow-rate throughout.

Preparation of glucose-silica

Glucose-silica was prepared as previously described [2]. However, in order to obtain glucose-silica consistently Cd–ninhydrin [6] negative at room temperature, we always carried out the reaction twice. Glucose-silica after the first reaction was washed with water only, and after the second reaction was first washed with acetone, then with water. All silica-based supports used were 7 μm Macherey–Nagel Nucleosil silicas obtained from Alltech (Deerfield, IL, USA) and had 50 Å pores.

Column testing

Glucose-silica supports were packed into five 100×4.6 mm I.D. columns by Alltech. The mobile phase was buffer A (100 m*M* sodium sulfate, 20 m*M* sodium phosphate, pH 6.8) throughout. The same sensitivity was used to record chromatograms for a particular test mixture throughout. After testing in each period, columns were flushed with appropriate storage buffers for about ten column volumes and stored (see *Storage conditions* below). The time table for retesting was 1 month, 3 months, 6 months and 1 year after the initial tests.

Proteins with opposite extremes of isoelectric pH (p*I*), were used to test the columns. Neutral but relatively hydrophobic tryptophan (M_r 204) was used as a low-M_r marker for total included column volume (V_T). Proteins of various molecular masses were also included to assess their sensitivity to stationary phase matrix instability. The individual proteins were typically made up as 1 mg/ml stock solutions and various mixtures were then prepared. The mixtures were aliquoted for long-term uses to prevent problems associated with repeated freezing and thawing. Each protein was also injected individually onto the various columns to confirm the identity of each peak in a mixture. The injection volume was 10 μl and detection was by absorption at 220 nm throughout.

Storage conditions

The five storage conditions were as follows: (A) 10 m*M* sodium azide in buffer A at 4°C; (B) 10% methanol in buffer A at 4°C; (C) 100% methanol at 4°C; (D) buffer A alone at 4°C; (E) 10 m*M* sodium azide in buffer A at room temperature.

Chemicals and reagents

The mobile phase buffer contained sodium phosphate (Mallinckrodt, Paris, KY, USA) and sodium sulfate (Sigma, St. Louis, MO, USA). Chicken egg lysozyme (Lyz, M_r 14 000, p*I* 11), horse heart cytochrome *c* (Cyt c, M_r 13 000, p*I* 9.4), bovine serum albumin (BSA, M_r 66 000, p*I* 5), soybean trypsin inhibitor (STI, M_r 20 000, p*I* 4.5), and L-tryptophan (Trp) were from Sigma; L-arginyl-L-phenylalanine (Arg–Phe) was from Cyclo, Los Angeles, CA, USA. Porcine brain calmodulin (CaM, M_r 16 700, p*I* 4) was purified as described [7]. (M_r and p*I* of these proteins are from references 2, 7 and 8.)

RESULTS AND DISCUSSION

Of the five storage conditions tested over a year's time, all of the data fit one of two patterns: those conditions which maintained reasonable chromatographic performance for about 6 months and those which showed rapid degradation of column performance. All of the data need not be shown to illustrate these findings; rather the results of the best and worst storage conditions only are shown in Fig. 1A and B, respectively. Fig. 1A shows that refrigeration in buffer A containing sodium azide maintained reasonable chromatographic performance for about 6 months. Quite similar results were obtained for columns that were refrigerated with either 10 or 100% methanol although the former showed somewhat better stability than the latter (data not shown). The worst storage condition found is illustrated in Fig. 1B, room temperature storage in buffer A containing sodium azide. This condition was only marginally worse than storage in buffer A (without azide) under refrigeration which also gave rapid degradation of column performance. Thus, our results with glucose-silica column stability indicate that both

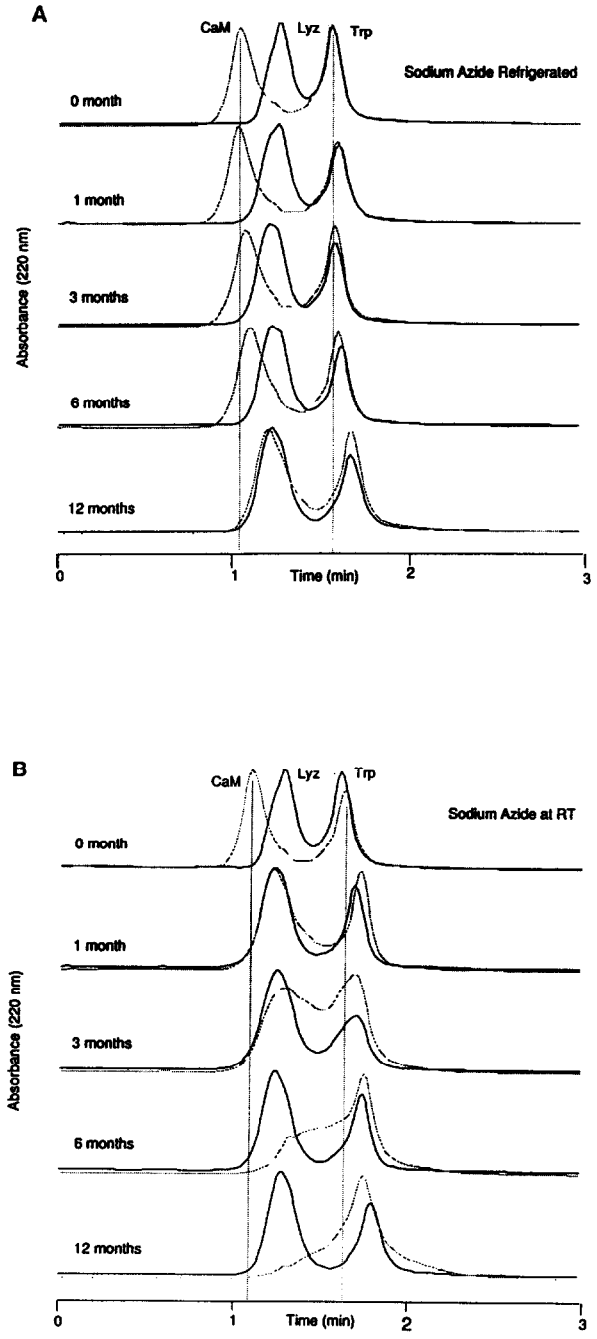

Fig. 1. Chromatographic behavior of acidic and basic proteins for columns stored in 10 m*M* sodium azide at 4 or 20°C. Two test mixtures: (1) Trp and acidic CaM seen as dotted line and (2) Trp and basic Lyz indicated as solid line, were injected onto the column at the indicated period. In Fig. 1B, RT = room temperature. (A) Column stored in 10 m*M* sodium azide at 4°C; (B) column stored in 10 m*M* sodium azide at RT.

refrigeration and inclusion of either methanol or sodium azide in the storage buffer are necessary to maintain relatively stable chromatographic performance; either treatment alone is not sufficient.

The data in Fig. 1A and B also demonstrate that the retention times of all tested substances were not affected the same during column failure. The retention times and peak shapes for Lyz were maintained under all storage conditions throughout a year. Calculations indicated less than 3% changes in retention times of Lyz for all columns. In contrast, CaM, an acidic protein of similar size to Lyz, exhibited peak shapes and retention times with more variation among columns stored differently. Fig. 1A shows that CaM peak shapes were reasonably constant for about 6 months for columns stored refrigerated in 10 m*M* sodium azide (Fig. 1A), 10% methanol or 100% methanol (data not shown). However, columns stored in buffer containing sodium azide at room temperature (Fig. 1B) or in refrigerated buffer alone (data not shown) gave CaM peak shapes characteristic of column degradation within 1 month. The column that gave the largest changes in the CaM peak was the column stored at room temperature (Fig. 1B) even though sodium azide was included as an anti-bacterial agent.

Fig. 2 shows that the chromatographic behavior of BSA, a relatively large acidic protein, was less affected under all storage conditions throughout a year. This result can be contrasted with the greater variation seen with the smaller acidic proteins CaM (Fig. 1) and STI (see below). The likely cause of BSA's stable behavior is that its large size excludes it from the silica pores where more of the changes related to column stability are occurring.

The retention times of Cyt c (Fig. 2), a small basic protein, were not significantly affected, in agreement with the data obtained for Lyz (Fig. 1). Both Cyt c and Lyz are basic and have similar M_r. Small, hydrophobic markers were actually more sensitive to column changes. The geometric volume, V_T, of the 100×4.6 mm columns used is 1.66 ml, which at this flow-rate corresponds to a retention time of 1.66 min. For gel-filtration chromatography, all species should

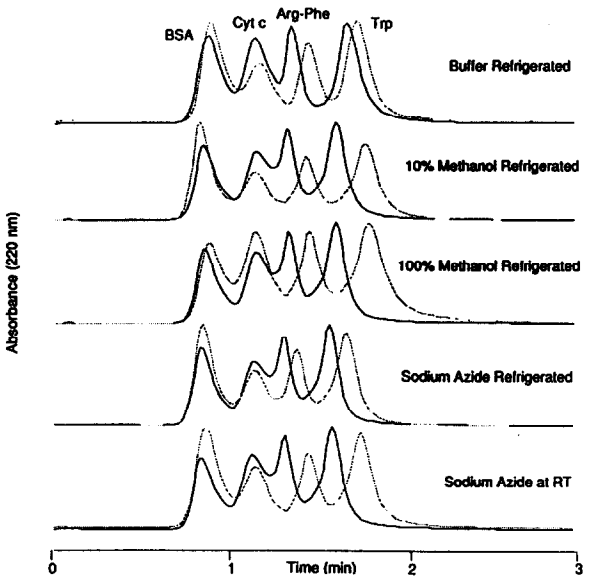

Fig. 2. Chromatographic behavior of a four-component mixture. Chromatograms were overlaid so that one can compare chromatographic changes before (solid line) and after (dotted line) a year of storage under the different conditions shown.

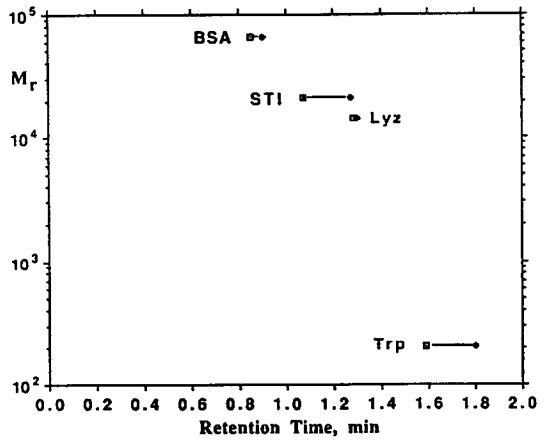

Fig. 3. Dynamic stability of glucose-silica. STI was used as an alternative source of low-M_r acidic protein for this study. The open squares represent day 1 (except for STI which began at day 2) and the closed diamonds represent day 7. 0.05% Sodium azide was added to the mobile phase buffer for the study. The column was stored overnight in this buffer at 4°C.

elute with smaller retention times than V_T. In general, as these columns began to fail, retention volumes of Trp, a small hydrophobic molecule, became greater than V_T. Arg–Phe, a basic, hydrophobic dipeptide, exhibited similar behavior (Fig. 2). These results indicate that as these glucose-silica columns begin to fail, other chromatographic separation modes begin to operate.

The increases in retention times throughout the year shown in Figs. 1 and 2 are not due to changes in the chromatograph over the year. Flow-rates of pumps can be affected by wear, tubing lengths can change, etc. and all of these could affect measured retention times. However, these factors were controlled for and do not account for the differences shown. The changes shown are due to time-dependent changes in the columns themselves.

Fig. 3 shows the results of a dynamic stability study of glucose-silica. For this study, a column was subjected to continuous chromatography each working day for a week with 0.05% sodium azide at room temperature, and the changes that occurred in retention times are illustrated. The greatest changes occurred with the small acidic protein (STI) and Trp, whose retention times were significantly increased throughout. The retention time of Lyz, a basic protein, was constant throughout. BSA, though also an acidic protein, was not much affected probably because of its large molecular size relative to the 50 Å pores. This observation and that of Fig. 2 for BSA suggest that totally excluded proteins are less susceptible to stationary phase changes in HPLC gel-filtration columns. While our long-term study of glucose-silica was necessary to determine the effects of a variety of storage conditions on stability, the data in Fig. 3 show that a dynamic stability study can be used to more rapidly detect unstable columns. This was useful in other studies where we have found that chemically modifying glucose-silica increases its stability [9].

Overall, Figs. 1–3 demonstrate that the observed chromatographic changes of glucose-silica over time were mainly consistent with the increasing presence of cationic and hydrophobic interactions on the support surface as storage is prolonged. This was detected in our study by the changing chromatographic behavior of acidic CaM and STI, the basic and hydrophobic Arg–Phe, and Trp throughout a year. CaM appeared

to bind cationic groups in an ion-exchange interaction with degraded columns.

The mechanism by which these changes occur is not at all certain; however, it seems likely that microbial contamination may be the cause. Removing glucose from glucose-silica would expose the underlying propylamine layer on the support surface, causing the observed increased retention times of CaM and STI. Increased retention of Arg–Phe and Trp suggests that hydrophobic interaction also accompanies column degradation.

Our data suggest that storing columns in sodium azide or methanol at 4°C was effective in maintaining the original chromatographic behavior for glucose-silica columns throughout at least 6 months of storage. This is based mostly on the observations of chromatographic peak shapes and retention times of separated mixtures. As new chromatographic media are synthesized with the goal of biocompatibility, the stability of these materials must be of special concern. Indeed, we have also found glycidiol-silica columns (*e.g.*, Alltech's Macrosphere GPC) to degrade within about 6 months when used daily for chromatography of CaM (unpublished results). The results with glucose-silica suggest that storage under refrigeration and inclusion of either 10 m*M* sodium azide or 10% methanol in mobile phases may be effective methods to prolong the useful lifetime of biocompatible columns. Refrigeration of these columns was also shown to be important, probably because it significantly slowed down microbial growth inside the glucose-silica columns; methanol- and NaN_3-resistant organisms may be responsible for this requirement.

Our data also suggest that mixtures used to test column performance should contain as many diverse kinds of proteins and other biochemicals as is feasible. For example, the results obtained with a test mixture containing only neutral and basic proteins may not be relevant to an investigation involving acidic proteins, testing with large proteins may not reveal changes which occur as readily as proteins which penetrate and interact with porous surfaces, etc.

This study demonstrates effective storage methods, which prevent the degradation of glucose-silica columns for about 6 months. However, the overall instability of glucose-silica observed in this study limits its usefulness. Since glucose-silica was shown to possess many excellent chromatographic properties [2], this instability suggests that while the approach of making polyhydroxylated silica coatings is a good one, these coatings will need to be designed in the future to enhance stability. We have tried the chemistry with maltose instead of glucose but found that such coating with disaccharide was inferior probably because of lower coupling efficiency under the same conditions we used to synthesize glucose-silica (unpublished results). Huisden *et al.* [10] recently also coupled maltose to aminopropyl-silica and cross-linked maltose on the surface. This treated maltose-silica appears to be more stable dynamically than glucose-silica. However, we believe that the proper storage conditions reported in this note may also be applied to prolong its static stability.

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