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Synthesis and characterization of highly stable polymer-coated aminosilica packing material for high-performance liquid chromatography*

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ABSTRACT

A synthetic procedure leading to an amino-bonded stationary phase for high-performance liquid chromatography was developed. The silica surface was first coated with a silicone polymer monolayer and an amino functionality was subsequently attached to the polymer layer. The polymer-coated amino-bonded phase showed improved stability and separation in the analysis of carbohydrates and nucleotides over conventional aminopropylsilylated silicas.

INTRODUCTION

Aminopropylsilylated silica is one of the most commonly used column packings for the analysis of carbohydrates [1], ionic compounds and other biologically important substances [2]. However, conventional aminopropylsilicas still have several problems. In general, aminopropylsilicas are chemically unstable and the lifetimes of packed columns are relatively short [3]. One of the reasons for the decrease in column efficiency is thought to be gradual dissolution of the primary amino phase in aqueous mobile phases [4]. Another complexity is that the three-dimensional structure of the aminopropyl phase formed on the silica surface varies depending on the reaction conditions owing to the multi-functionality of 3-aminopropyltriethoxysilane, the most commonly used silanizing reagent [5], which makes it difficult to obtain reproducible chromatographic separations.

Several efforts to prepare improved amino-bonded phases have been reported. Cationic polymers whose amino groups were cross-linked with diepoxides showed good reproducibility during continuous operation for several months [6,7].

The use of (3-aminopropyl)diisopropylethoxysilane, an alternative silanizing reagent, has been reported [8]. The two bulky isopropyl groups of the reagent were assumed to protect the silica surface against hydrolysis. Aminopropylsilica prepared by gas-phase silylation also gave improved results with regard to the peak shape of saccharides [9], although the durability of the column was still uncertain.

As mentioned above, the initial instability of silica-based aminopropyl-bonded stationary phases appears to be due to the gradual desorption of the respective silane, while further changes in the chromatographic properties during the operation seem to be caused by deactivation of the surface with impurities in the mobile phase. A simple and effective way to prevent

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degradation caused by mobile phases is to use a protective column between the pump and the injection valve that is packed with the same sorbent. Such a protective column allowed the main column to be used for several months without any chromatographic change in a certain application [10].

In spite of these efforts, improvements in the chromatographic stability of primary amino-bonded phases still remains a major issue. So far, none of the commercially available amino-propylsilica columns seems to have reached an acceptable level in terms of its life span and separation efficiency.

A novel polymer-coating technique to form reactive polymethylsiloxane films on metal oxides by chemical vapour deposition of 1,3,5,7-tetramethylcyclotetrasiloxane (H4) was developed by Fukui et al. [11]. Ohtsu and co-workers [12,13] utilized this polymer coating technique to prepare a polymer-coated C_{18} silica packing. The polymer-coated C_{18} silica showed high chemical stability even in alkaline mobile phases [12] and separation characteristics similar to those of conventional octadecylsilylated silica (ODS) phases [14]. The polymer-coated C_{18} silica seems to have a more homogeneous surface than another type of polymer-coated silica phase on which the bulky C_{18}-modified silicone polymer was directly anchored [14,15]. Polymer-coated C_{18} packings, prepared from high-purity silica, also showed an excellent performance in the analysis of protonated amines and chelating compounds, which are normally considered difficult to analyse owing to their undesirable secondary interaction with the silica surface [16].

In this paper, an alternative way to develop a silica-based amino-bonded phase using the above-mentioned polymer coating technique is described. The synthetic procedure, the stability of the material and applications to various biologically important substances are discussed.

EXPERIMENTAL

Materials

High-purity silica (Shiseido, Tokyo, Japan) (particle size 5 μm, pore diameter 80 A, specific surface area 400 m^2/g, metal impurities <5 ppm) was used as a starting material for the polymer-coated amino-bonded phase. 1,3,5,7-Tetramethylcyclotetrasiloxane (H4), the silicone monomer used for the polymer coating, was purchased from Toshiba Silicone (Tokyo, Japan). All the reagents and solvents used to synthesize the packing were of special grade from Nacalai Tesque (Kyoto, Japan), and were used as received. Carbohydrates, sugar alcohols, allantoin and fat-soluble vitamins used as standard compounds were obtained from Nacalai Tesque. Oligosaccharides were obtained from JASCO (Tokyo, Japan). Nucleotide monophosphates were purchased from Sigma (St. Louis, MO, USA). Acetonitrile used for the mobile phases was of HPLC grade from Nacalai Tesque. Potassium dihydrogenphosphate and phosphoric acid to adjust the pH of the mobile phase were of special grade from Nacalai Tesque. Water was purified with a Milli-Q system (Nihon Millipore Kogyo, Tokyo, Japan). A Nucleosil $5NH_2$ column (250 mm x 4.6 mm I.D.) was purchased from Macherey-Nagel (Düren, Germany) and was used for comparative studies as described later.

Synthesis of amino phase

The H4-coated silica was prepared according to the method of Fukui et al. [11]. The high-purity silica gel (100 g) was placed in contact with the vapour of H4 (40 g) at 100°C for 48 h under a nitrogen atmosphere. The H4 molecules were deposited on the silica surface and became polymerized. The measured thickness of the homogeneous polymer layer was *ca.* 7 A, which corresponds to that of a monolayer. The silicone polymer formed still had a high proportion of reactive SiH groups (2.30 mmol/g), sufficient for the subsequent modification. Ally1 glycidyl ether (AGE) (100 g) was reacted with the SiH groups of the polymer-coated silica (100 g) in refluxing 2-propanol (200 ml) for 5 h under a nitrogen atmosphere in the presence of hexachloroplatinic acid (50 mg). When the hydrosilylation reaction was complete, the solvent mixture was removed with a glass filter and the epoxysilica trapped on the filter was washed thoroughly with 2-propanol and methanol. The epoxysilica was dried *in*

vacuo for 5 h at 150°C to remove the residual AGE completely. Aqueous ammonia (100 g) or polyamines [$NH_2(CH_2CH_2NH)_nH$, (n = 1–5), 100 g] were added to the epoxysilica (100 g) and the mixture was heated in refluxing 2-propanol (200 ml) for 5 h under a nitrogen atmosphere. After the removal of the solvent, the aminosilica was washed with 2-propanol several times and then with methanol and water. The wet aminosilica was stirred in a mixture of formaldehyde (20 ml) and water (100 ml) for 30 min at room temperature, then droplets of 0.1% sodium carbonate solution were added to the slurry in order to adjust the pH to 9.0 and the slurry was stirred for an additional 30 min at room temperature. After filtering off the solvent, the cross-linked aminosilica was washed with water and methanol. The modified silica was dried *in vacuo* for 5 h at 120°C.

Characterization of polymer-coated aminosilica (NH_2-silica)

The modification density of the polymer-coated NH_2-silica was calculated from the nitrogen content measured by elemental analysis. The polymer-coated NH_2-silica (3.5 g) was dispersed in 30 ml of slurry solvent [cyclohexanol–methanol (40:60)] and the slurry was packed into a stainless-steel column (250 mm × 4.6 mm I.D.) with 80 ml of methanol and 80 ml of water at a constant pressure of 400 kgf/cm^2.

The durability of the polymer-coated NH_2-silica column was evaluated by comparing the decrease in the retention of maltose using a standard mobile phase [acetonitrile-water (75:25)] with that for the Nucleosil $5NH_2$ column. Further, chemical stability of the polymer-coated NH_2 column was evaluated by checking the retention time and peak shape of carbohydrates, which were measured after two series of alternating 4-h flows (1.0 ml/min) of two different mobile phase conditions: (1) acetonitrile-water (75:25), pH 2, adjusted with phosphoric acid, and (2) acetonitrile-water (75:25), pH 11, adjusted with aqueous ammonia. This flow programme of the mobile phases is given in detail in Fig. 2.

In addition to the fundamental performance in the separation of mono- and disaccharides, oligosaccharides, sugar alcohols, allantoin and fat-soluble vitamins, the characteristics of the new amino-bonded phase as a weak anion exchanger was investigated by observing the retention behaviours of the five standard nucleotides at different pH values and buffer salt concentrations.

Equipment

The HPLC system consisted of a DGU-4A degasser, an LC-9A pump, a SIL-6B autoinjector, an SPD-6AV UV detector, an SCL-6B system controller and a C-R4AX data processor (all from Shimadzu, Kyoto, Japan).

RESULTS AND DISCUSSION

The density of AGE molecules (spacer molecules) attached to the polymer-coated silica by hydrosilylation was 0.7 mmol/g. Various compounds possessing primary and secondary amino groups could be attached to these spacer molecules (Table I). In a preliminary experiment, the epoxy groups of the spacer molecules were directly converted into primary amines by a simple reaction with aqueous ammonia. The density of primary amino groups formed in this reaction was 0.4 mmol/g. Although this product could be used as an amino-bonded stationary phase, the measured retention of maltose was not large enough for practical use (Table I). The retention of maltose increased when ammonia was replaced with larger polyamines [$NH_2(CH_2CH_2NH)_nH$ (n = 1–5)]. As shown in Table I, the retention of maltose increased as the degree of polymerization (n) increased. When pentaethylenehexamine (n = 5) was used, the modification density (sum of amino and imino groups) increased to 2.5 mmol/g and the retention of maltose became sufficient for further applications.

A column packed with the pentaethylenehexamine-modified silica, however, did not show a largely improved lifetime, which had been expected owing to the silicone polymer coating. A cross-linking procedure appeared to be one of the ways to improve the durability of the amino-bonded phase [6,7]. Three cross-linking reagents were tried for the pentaethylenehexamine-

TABLE I

POLYAMINE ADDITION AND RETENTION OF MALTOSE

Amine added[a]	C (%)	N (%)	Imino and amino groups (mmol/g)	$k'_{maltose}$ [b]
Aqueous ammonia	7.6	0.6	0.4	1.15
Ethylenediamine	8.2	1.8	1.3	2.32
Triethylenetetramine	10.4	2.4	1.7	4.61
Tetraethylenepentamine	12.2	3.1	2.2	5.43
Pentaethylenehexamine	12.8	3.5	2.5	6.22

[a] 0.72 mmol/g of epoxy group was added to the SiH moiety of silicone polymer film on the 80 Å pore silica gel (400 cm^2/g) prior to polyamine addition.

[b] Dead volume was determined by the retention volume of acetonitrile which was not considered to be retained in the NH, column.

modified phase; glycerol diglycidyl ether, epichlorohydrin and formaldehyde. As expected, all of these reagents lengthened the lifetime of the column considerably. With these three reagents, however, the retention of maltose decreased by 80%, 47% and 39%, respectively. This retention decrease seems to be attributable to the increase in surface hydrophobicity caused by the cross-linking procedure. Among the three reagents, formaldehyde seems to be the best cross-linking reagent, providing a high stability and maintaining retention characteristics similar to those of conventional aminopropylsilicas.

Fig. 1 shows the life span of the polymer-coated NH, phase cross-linked with formaldehyde and that of the conventional Nucleosil $5NH_2$ aminopropylsilylated silica column. The capacity factor (k') and theoretical plate number (N) of maltose on the polymer-coated NH, column remained almost constant during 150 h of continuous use. On the other hand, the k' of maltose on the Nucleosil $5NH_2$ column decreased to 70% of the initial value after 100 h and serious tailing of the peak was observed. Hence the polymer-coated NH, column has a much longer life span than that of the conventional column.

Fig. 2 shows the change in the chromatogram for sugar analysis with the polymer-coated NH, column under extreme conditions with the cyclic

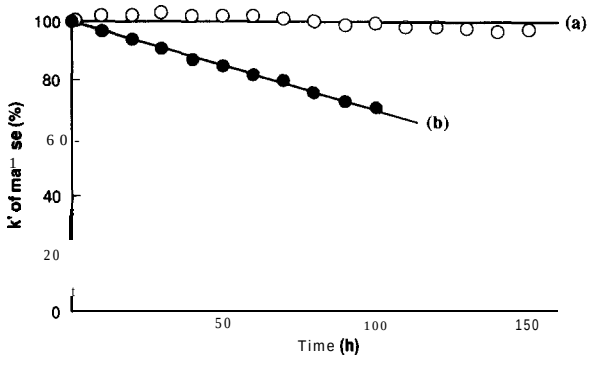


Fig. 1. Comparative degradation of silica-based NH, columns. Column: (a) polymer-coated NH,; (b) Nucleosil $5NH_2$. Column dimensions, 250 × 4.6 mm I.D.; column temperature, 40°C; mobile phase, acetonitrile-water (75:25); flow-rate, 1.0 ml/min; detection, refractive index (RI); sample, maltose.

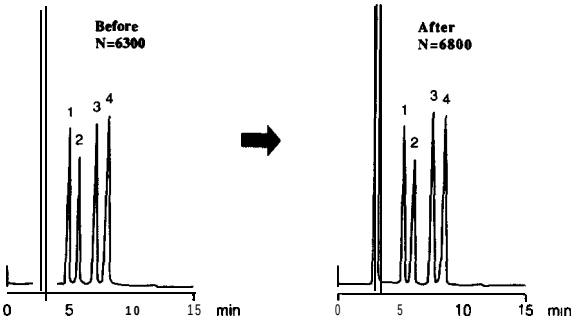


Fig. 2. Alteration of chromatogram of saccharides after cyclic test with acidic and basic mobile phases. Column, polymer-coated NH, (250 × 4.6 mm I.D.); temperature, 40°C. Mobile phase: acetonitrile-water (75:25), (1) pH 2 adjusted with H_3PO_4, (2) pH 11 adjusted with NH, solution and (3) pH not adjusted, with the following alternating cycle: (1) 4 h+(3) 1 h-,(2) 4 h+(3) 1 h+(l) 4 h-+(3) 1 h→ (2) 4 h→ (3) 6 h. Flow-rate, 1.0 ml/min; detection, RI. Sample: 1 = fructose; 2 = glucose; 3 = sucrose; 4 = maltose.

alternation of acidic (pH 2) and basic (pH 11) mobile phases. The peak shape and N value of maltose did not deteriorate significantly even after these extreme conditions. The hydrophobic silicone polymer film and cross-linked structure seem to prevent hydrolysis of the amino-bonded stationary phase.

The polymer-coated NH, column showed good performance in the analyses of mono-, di- (Fig. 3a) and oligosaccharides (Fig. 4), and sugar alcohols (Fig. 5). The overall retention of these

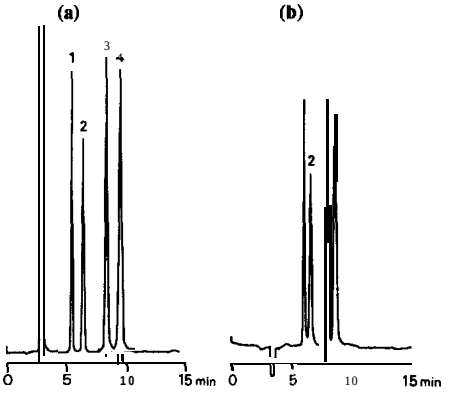


Fig. 3. Chromatograms of saccharides. Column: (a) polymer-coated NH,; (b) Nucleosil5 NH,. Column dimensions, 250 X 4.6 mm I.D.; column temperature, 40°C; mobile phase, (a) acetonitrile-water (75:25) and (b) acetonitrile-water (70:30); flow-rate, 1.0 ml/min; detection, RI. Sample: 1 = fructose; 2 = glucose; 3 = sucrose; 4 = maltose.

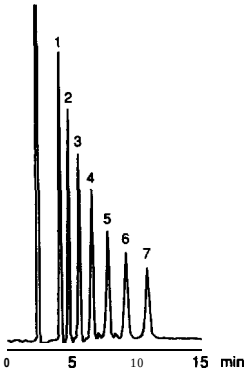


Fig. 4. Chromatogram of oligosaccharides. Column, polymer-coated NH, (250X4.6 mm I.D.); temperature, 40°C; mobile phase, acetonitrile-water (65:35); flow-rate, 1.0 ml/min; detection, RI. Sample: 1 = glucose; 2 = maltose; 3 = maltotriose; 4 = maltotetraose; 5 = maltopentaose; 6 = maltohexaose; 7 = maltoheptaose.

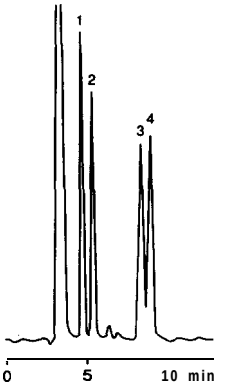


Fig. 5. Chromatogram of sugar alcohols. Column, polymer-coated NH, (250 x 4.6 mm I.D.), temperature, 40°C; mobile phase, acetonitrile-water (85:15); flow-rate, 1.0 ml/min; detection, RI. Sample: 1 = glycerine; 2 = pentaerythritol; 3 = D-sorbitol; 4 = mannitol.

standard analytes on the polymer-coated NH, column is slightly less than that on the conventional amino-bonded phase, probably owing to the increased hydrophobicity of the cross-linked polyamine. The overall retention of the standard analytes could be increased to the level for the conventional amino phase by increasing the acetonitrile content in the mobile phase by 5%. For instance, the retention of mono- and disaccharides on the polymer-coated NH, column with 75% acetonitrile in the mobile phase (Fig. 3a) was almost the same as that on the Nucleosil $5NH_2$ column with 70% acetonitrile (Fig. 3b). Note that the polymer-coated NH, column gave a better separation of mono- and disaccharides than did the Nucleosil $5NH_2$ column. It should be also noted that the large oligosaccharides ($M_r > 1000$) were separated with a relatively narrow peak width (Fig. 4), although these molecules were expected to be difficult to separate in this system having a small pore size (80 A) and a bulky structure of the cross-linked polyamine.

Allantoin is one of the most common medicinal ingredients in cosmetics and eyewashes. Allantoin is normally determined using an amino-bonded phase, because it is not retained in an ODS column owing to its hydrophilic nature. Allantoin could be retained on the polymer-coated NH, column (Fig. 6). Nico-

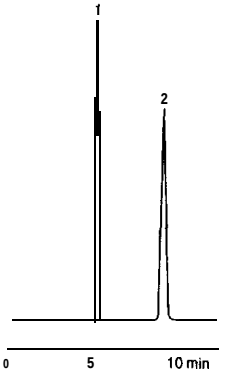


Fig. 6. Chromatogram of Allantoin. Column, polymer-coated NH, (250 × 4.6 mm I.D.); temperature, 40°C; mobile phase, 5 m*M* KH_2PO_4 (pH 2.0 adjusted with H_3PO_4) in acetonitrile-water (80:20); flow-rate, 1.0 mllmin; detection, UV (210 nm). Sample: 1 = allantoin; 2 = nicotinamide.

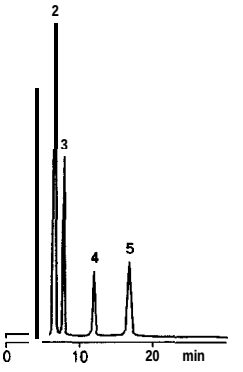
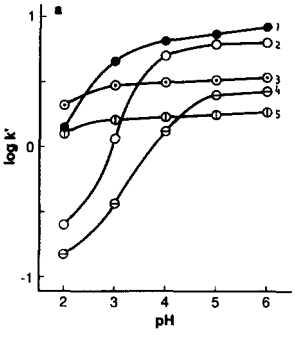


Fig. 8. Chromatogram of nucleotides. Column, polymer-coated NH, (250 x 4.6 mm I.D.); temperature, 40°C; mobile phase, 50 m*M* (NH,), HPO, (pH 3.0 adjusted with H_3PO_4); flow-rate, 1.0 mllmin; detection, UV (260 mn). Sample: 1 = 5′-CMP; 2 = 5′-AMP; 3 = 5′-UMP; 4 = 5′-IMP; 5 = 5′-GMP.

tinamide seems to be a suitable internal standard for the determination of allantoin under the mobile phase conditions shown in Fig. 6.

Standard fat-soluble vitamins were separated with the polymer-coated NH, column using hexane as the mobile phase, as shown in Fig. 7. Vitamin A palmitate and vitamin E acetate were separated as sharp peaks. The use of non-polar solvents, such as hexane, is not possible with certain polymer-based amino phases having a swelling nature.

Further, the polymer-coated NH, column was applied to the separation of nucleotide mono-



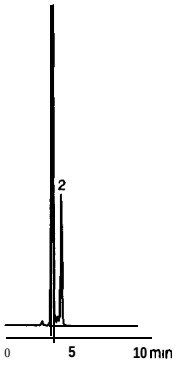


Fig. 7. Chromatogram of fat-soluble vitamins. Column: polymer-coated NH, (250 x 4.6 mm I.D.); temperature, 40°C; mobile phase, hexane; flow-rate, 1.0 mllmin; detection, UV (254 nm). Sample: 1 = vitamin A palmitate; 2 = vitamin E acetate.

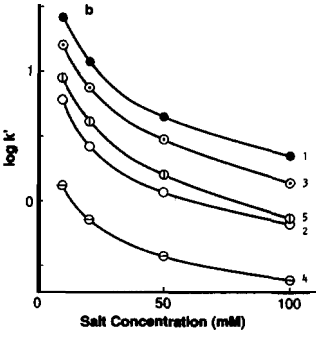


Fig. 9. Effects of (a) pH and (b) salt concentration on retention. Column, polymer-coated NH, (250 x 4.6 mm I.D.); temperature 40°C; flow-rate, 1.0 ml/min; detection, UV (260 nm). Mobile phase: (a) 50 m*M* $(NH_4)_2HPO_4$; (b) $(NH_4)_2HPO_4$ (pH 3.0). (1) = 5′-GMP; 2 = 5′-AMP; 3 = 5′-IMP; 4 = 5′-CMP; 5 = 5′-UMP.

phosphates (Fig. 8), for which a weak anion-exchange column has usually been used. The mobile phase was aqueous phosphate buffer. The retention of all the nucleotides decreased at lower pH (Fig. 9a) and with a higher salt concentration in the mobile phase (Fig. 9b). This type of retention behaviour of acidic solutes can be explained as an anion-exchange effect. The function as an anion exchanger of the polymer-coated amino-bonded phase seems to be due to the tertiary amine moiety of the cross-linked polyamine.

CONCLUSIONS

A polymer-coated NH, silica packing was developed by coating silica with a silicone polymer homogeneously and subsequent modification leading to a cross-linked polyamine phase. The polymer-coated NH, column showed a longer life span than that of a conventional silica-based NH, column owing to the existence of a protective silicone polymer film and a cross-linked polyamine. It is applicable not only to the analysis of saccharides, sugar alcohols, allantoin and fat-soluble vitamins, but also to the anion-exchange separation of nucleotides.

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