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CXXIX☆. Ceramic-based particles as chemically stable chromatographic supports

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ABSTRACT

Porous zirconia based particles have been modified using different derivatisation procedures. The modified particles were characterised in terms of their accessible surface areas and degree of surface coverage of the bounded or physicoated phases utilising the strong and specific adsorption of phosphate ions to the zirconia surface. The hydroxyl group density was determined by a ^{1}H NMR technique.

The particles were modified by immobilising different silanes to introduce either hydrophobic ligands or reactive groups onto the zirconia surface. In the latter case, various ligands were then covalently attached to the activated supports. Using this type of modification, n-octadecyl- (C_{18}), carbohydrate- and Cibacron Blue F3GA-modified zirconia particles were produced. Furthermore, polymeric coated particles were prepared either by using polybutadiene or by cross-linking the carbohydrate modified sorbents.

The pH stability of the different sorbents were determined in batch experiments and under chromatographic conditions. The leakage of ligands was monitored by UV absorption and by employing radioactively labelled ligands. The performance of the C_{18} reversed-phase modified zirconia in packed columns was also used as an indicator of changes in the surface chemistry following pH stability tests. The experimental results indicate that the Cibacron Blue F3GA dye-modified sorbent was stable up to pH 10.5, the C_{18} reversed-phase packing up to pH 13 and the carbohydrate-bonded phase up to pH 12. These investigations substantiate the favourable chemical and physical characteristics anticipated for surface modified zirconias for potential use as chromatographic adsorbents.

INTRODUCTION

Until now there have been two major families of support materials competing as sorbents in HPLC [1]. THe first family of sorbents is based on organic polymers and the second is based on silica. The properties of each of these materials have advantages but they also have certain disadvantages. Both types of support materials can be produced in a wide and controlled range of particle sizes, porosities and pore diameters. Polymeric based particles have a high chemical stability over a wide pH range and are fairly inexpensive to produce but they lack physical strength at high pressures and tend to be affected by organic solvents and high temperature resulting in deformation of the structure of the pores and particles.

Silica-based particles show a high physical strength and can easily be modified. Accordingly, a wide range of modified silica packings are commercially available [2,3]. Although silica-

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☆ For Part CXXVIII, see ref. 34.

based sorbents can be used with a wide range of solvents, their major disadvantage is their low stability at pH values above 8.2. Attempts to improve the pH stability by stabilising the surface with zirconia and alumina [4,5] have been made with partial success. Alternatively, coating the particles with a polymeric layer [6,7] or with hindered silanes [8] has proved attractive, but once the polymeric layer or silane is damaged the particles degrade very rapidly.

Various attempts have been undertaken to utilize the known high chemical stability of zirconia and other metal oxides such as titania or alumina for chromatographic purposes. The preparation of zirconia particles resulting from a sol-gel process have been described by Trüdinger et al. [9] and Yoldas [10] and results on the application of these modified and non-modified particles presented. In several studies zirconia particles have been used untreated as normal-phase sorbents [11–13], whilst other investigations have used polymeric coated particles [14–16]. Recently, the high affinity of zirconia for fluoride and phosphate ions has been used to cover the zirconia surface in order to suppress non-specific interactions with biomolecules [17–20]. The amount of surface coverage with these ions however is pH dependent and therefore changes under various conditions. Modifications of zirconia with organosilanes have been reported to be less stable than the equivalent silica support [14]. However, the results presented in the present series of investigations demonstrate that zirconia can be modified with organosilanes in the way described to produce sorbents superior to silica [21]. Since it is possible with zirconia-based particles to combine the advantages of density, mechanical strength and chemical stability, we were particularly interested to explore further procedures to chemically produce modified surfaces suitable for biochromatography. In this paper we present results that show that these particles are at least as physically stable, and as easy to modify, as silica particles. The experimental results also confirm that surface modified zirconia sorbents are also compatible with most organic solvents and show a remarkable stability at high pH values.

MATERIALS AND METHODS

Chemicals and support material

Two types of ceramic particles based on zirconia were used in these investigations. One type (referred to as PDZ later in the text) was prepared as part of the bioceramics programme at the Centre for Bioprocess Technology, Monash University, while the other was donated by the 3M Company, St. Paul, MN, USA and used as a comparison to the PDZ materials.

The 3-mercaptopropyl-triethoxysilane, poly-butadiene (M_r 4500), imidazole and dicumyl peroxide were supplied by Sigma, Cibacron Blue F3GA was supplied by Serva, Germany and octadecyldimethylchlorosilane was supplied by Wacker-Chemie, Germany. All other chemicals were of analytical or chromatographic grade. The columns used were supplied by Bishoff, Germany (38 mm long × 10 mm I.D.) and by SGE, Australia (100 mm long × 2 mm I.D.).

CHARACTERISATION OF THE PARTICLES

Determination of the surface area by adsorption of phosphate ions

The free zirconia surface area can be measured by the amount of phosphate ions that is able to bind onto the surface via strong interactions with the Lewis acid (zirconol) sites present on the zirconia surface. For this determination, a phosphate solution of known concentration is used. An aliquot of this solution was stored as a standard solution for the determination of the control levels of phosphate concentration. To other solution aliquots, a known weight of zirconia particles (50 mg/ml) was added and the suspension shaken overnight at room temperature. The solid particles were removed by filtration, and the phosphate concentration of the supernatant measured.

These measurements were achieved using the Molybdenum Blue method described by Vogel [22], which was modified to be applied in a microtiterplate. The principle of this method involves the interaction between orthophosphate

and molybdate ions to form molybdophosphoric acid in acidic solution, which can be selectively reduced by hydrazine sulphate to form molybdenum blue, a compound of uncertain composition. This complex can be measured spectrophotometrically at its absorption maximum at 820 nm.

Reagents

Molybdate solution. A 12.5-g amount of sodium molybdate was dissolved in 500 ml 5 *M* sulphuric acid.

Hydrazine sulphate solution. A 1.5-g amount of hydrazine sulphate was dissolved in 1 l water.

Standard phosphate solution. Potassium dihydrogenphosphate was dissolved in water at concentrations between 0.1 and 10 mg/l.

The assay was performed in microtiterplates (PS microplate F-form, Greiner, Germany) and measured with a Titertec Platereader obtained from Flow Instruments, USA.

Procedure

To obtain linearity in the microtiterplate assay, the concentration of phosphate ions in the sample should be smaller than 12 mg phosphate per liter. In the micro titerplate well, 50 μl of sample at neutral pH was mixed with 10 μl molybdate solution and 4 μl hydrazine sulphate solution and diluted to 100 μl total volume. The mixture was heated in a boiling water bath for 10 minutes and then cooled rapidly. Due to the fact that no filter was available for the Titertek plate reader at the 820 nm band the optical absorption of the supernatant samples from naked and

derivatised zirconias was measured in a microtiter plate together with reference to different dilutions of the standard phosphate solution as calibration. Table I shows the results of phosphate adsorption on different modified and unmodified zirconia support materials.

Determination of the hydroxyl group density by ^{1}H NMR of D-trifluoroacetic acid

The hydroxyl group density on the zirconia surface is measured by ^{1}H NMR, based on a method by Holík and Matějková [23].

To obtain a calibration curve, 10, 8, 6, 4 and 2 μl water was mixed in 1 ml D-trifluoroacetic acid (D-TFA). The underivatised zirconia samples, 0.5 g in the case of the 3M material and 1.0 g in case of the PDZ zirconia, were dried at 110°C, suspended in 1 ml D-TFA and left for 1 h. Aliquots of 500 μl were removed and filtered prior to adding to a NMR tube and ^{1}H measurement on a Bruker AM 300 at 300 MHz, locked on the deuterium signal of the D-TFA. The temperature was 293 K. *p*-Xylene (3%, v/v) was used as an internal standard.

Hydrothermal treatment to increase the hydroxyl group density on the zirconia surface

To increase the hydroxyl group density on silica surface three methods are commonly used: (i) treatment with nitric acid [24], (ii) treatment with nitric acid under elevated temperatures and (iii) hydrothermal treatment [25].

In the case of the zirconia particles the first

TABLE I

AMOUNT OF PHOSPHATE IONS ADSORBED ON MODIFIED AND NON-MODIFIED ZIRCONIAS

Material (1.0 g)	Amount of phosphate adsorbed (mg)	Free surface area (%)
3M, not modified	1.53	100
3M, Cibacron Blue modified	0.39	25.5
3M, Glucose modified	0.67	43.8
PDZ, not modified	0.158	100
PDZ, Cibacron blue modified	0.036	22.8
PDZ, C$_{18}$ modified	0.023	14.5

two methods showed no significant effect on the hydroxyl group density. Therefore, the hydrothermal treatment was applied to achieve a higher amount of reactive hydroxyl groups for the subsequent chemical modifications. In particular, the zirconia particles were treated in an autoclave in a water steam atmosphere at 423 K for 6 h.

CHEMICAL MODIFICATION OF THE ZIRCONIA SUPPORTS

Modification with mercaptosilane and Cibacron Blue F3GA

The modification of the zirconia with Cibacron Blue was performed in two steps as illustrated in Fig. 1. First, the zinconia particles were activated with 3-mercaptopropyltrimethoxysilane (MPT-silane) and then derivatised with Cibacron Blue F3GA. To couple the MPT-silane to the zirconia surface, the particles were suspended in aqueous nitric acid, pH 3.5. The MPT-silane (8 μmol silane per m^2 surface area of the particles) was added and the suspension was shaken at 363 K for 3 h. The derivatisation with Cibacron Blue F3GA (2 μmol triazine dye per m^2) was performed at 333 K in 0.1 M sodium carbonate buffer pH 8.0 containing 0.5 M NaCl overnight.

The amount of MPT silane necessary to modify the particles was calculated from the product of the specific surface area, the amount of zirconia, the hydroxyl group density (about 8 μmol/m^2) and the molecular mass of the MPT-silane. Because of steric reasons only half of the hydroxyl groups are accessible to the MPT-silane. Therefore, using the value of 8 μmol/m^2 as the accessible hydroxyl group density results in a twofold excess of the MPT-silane. Higher amounts of the MPT-silane should be avoided

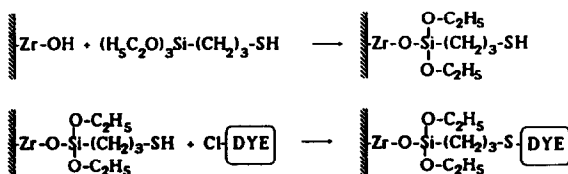because multilayer coverage can occur leading to closure of some pores.

Immobilisation of the Cibacron Blue F3GA dye is not limited by the number of reactive sites on the particle surface but by the size of the dye molecule. Again, a twofold excess was used for the reaction on the basis that the maximum amount of dye able to bind to the support is about 1 μmol/m^2. After the reaction was completed, the dye sorbents were washed with 100 ml each of water, and 2-propanol [26].

Modification with octadecyldimethylchlorosilane

The modification was performed with octadecyldimethyl chlorosilane (ODS) in anhydrous toluene, using imidazole as a catalyst (Fig. 2). The toluene was stored over sodium metal and freshly distilled before use. To remove physically adsorbed water from the zirconia surface the particles were treated at 440 K in vacuum overnight. The zirconia particles (2 g) were suspended in 50 ml toluene, equimolar amounts of imidazole and the ODS-silane were added, the mixture treated in an ultrasonic bath for five minutes and then heated under reflux for 6 h. The ODS-silane was added in an eightfold excess assuming that the maximum ligand density which could be achieved would be about 4 μmol/m^2. To prevent grinding of the particles the use of a magnetic stirrer was avoided during the reflux. After the reaction was finished, the sorbent material was washed with toluene, 2-propanol and water.

Modification with polybutadiene

The zirconia particles were modified using two different amounts of prepolymerised polybutadiene (PBD), resulting in sorbents with different thickness of the polymeric layer [6,7]. For the low carbon loading, the amount of PBD was calculated to be 0.5 mg/m^2 and for the high

Zr-OH + (H$_5$C$_2$O)$_3$Si-(CH$_2$)$_3$-SH   ⟶   Zr-O-Si(-(CH$_2$)$_3$-SH, O-C$_2$H$_5$, O-C$_2$H$_5$

Zr-O-Si-(CH$_2$)$_3$-SH + Cl⟨DYE⟩   ⟶   Zr-O-Si-(CH$_2$)$_3$-S⟨DYE⟩, O-C$_2$H$_5$, O-C$_2$H$_5$

Fig. 1. Modification of zirconia with Cibacron blue F3GA.

Zr-OH + Cl-Si-(CH$_2$)$_{17}$-CH$_3$ ⟶   Zr-O-Si-(CH$_2$)$_{17}$-CH$_3$, CH$_3$, CH$_3$
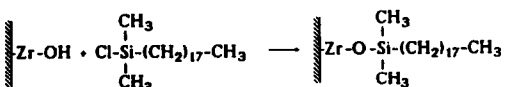
Fig. 2. Modification of zirconia with octadecylsilane.

carbon loading 1.5 mg/m². The PBD and the dicumylperoxide (DCP) were dissolved in dry pentane and the dried zirconia particles were added. The suspension was shaken at room temperature overnight. The pentane was removed under vacuum and the coated particles were heated to 333 K under vacuum for 12 h. The final step was heat treatment at 473 K under nitrogen atmosphere for 4 h to cross-link the coating.

Modification with aminosilane and carbohydrates

The modification of zirconia with carbohydrates was based on procedures developed for silica-based sorbents [27–29] (see Fig. 3). To 1 g of zirconia (dried overnight under vacuum at 453 K) suspended in 50 ml anhydrous toluene, 3-aminopropyltrimethoxysilane (8 μmol/m² surface area) was added corresponding to a twofold excess compared to the accessible hydroxyl

Fig. 3. Modification and cross-linking of zirconia with carbohydrates.

group density on the zirconia surface (determined as described above for the modification with 3-mercaptopropyltriethoxysilane). The reaction was performed by heating the suspension under reflux for 6 h. After completion, the particles were extensively washed with toluene, 2-propanol, 10 mM HCl and water.

Glucose or maltose was coupled to the aminopropyl-zirconia in a 50 mM sodium carbonate buffer pH 6.8. An estimated tenfold excess of glucose or maltose was used for the coupling, which was performed by shaking the suspension at 333 K overnight. An equimolar amount of sodium cyanoborohydride, relative to the amount of carbohydrate present, was included to reduce the Schiff's base that is formed. After the reaction was completed the particles were washed and suspended in acetone prior to cross-linking the carbohydrate chains with butadienediepoxide. For the cross-linkage step, different amounts of the diepoxide ranging from 10 to 100 μl per gram of zirconia particles were used. The cross-linkage reaction was performed for two hours at ambient temperature with boronitrifluoride diethyletherate as catalyst.

Any remaining epoxide rings were opened either by acid treatment [30] or by deactivation with ethanolamine [31]. The derivatised sorbents were either used without any further treatment or modified with Cibacron Blue F3GA. For the modification with the triazine dye, the particles were suspended in 100 mM sodium carbonate buffer pH 9.5 containing 0.5 M NaCl and an excess of Cibacron Blue F3GA (ca. twofold) was added. The reaction was performed at 333 K overnight, after which the particles were washed with water and 2-propanol.

The amount of coupled aminosilane was determined by elemental analysis and the amount of coupled glucose was estimated from the difference between uncoupled glucose in the supernatant and the amount of glucose added to the "coupling solution". The result indicates that at least 97% of the amino groups were derivatised by glucose units. This result was supported by the fact that aminosilyl-derivatised zirconia following chemical modification with glucose does not give any colour reaction with picrylsulphonic acid.
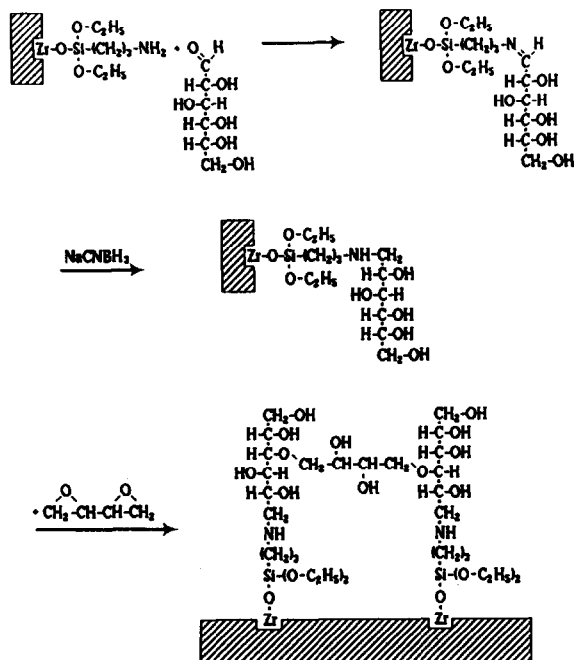
Modification with 3-glycidoxypropyltrimethoxysilane (Glymo)

Two different modification procedures were used with Glymo-silane; (A) modification under anhydrous conditions and (B) modification in aqueous solution at acidic conditions. Under anhydrous conditions, Glymo-silane will monomerically modify the zirconia, while at acidic conditions a polymeric layer will be formed.

(A) Modification under anhydrous conditions

A 2-g amount of zirconia was rehydroxylated with hydrothermal treatment at 423 K for 6 h, after which the particles were dried under vacuum at 453 K overnight. The particles were suspended in 50 ml anhydrous toluene, 17 mg Glymo-silane and 15 mg imidazole as a catalyst were added and the suspension was heated under reflux for 6 h. The modified particles were washed with toluene, 2-propanol and water.

(B) Modification under aqueous conditions of acidic pH

The zirconia particles were rehydroxylated as described under method A and modified according to a procedure described by Regnier and Noel [30]. A 2-g amount of zirconia was suspended in 20 ml of a 10% solution of the Glymo-silane in water adjusted to pH 3.5 with nitric acid. The suspension was heated at 363 K for 2 h, after which the particles were washed with water to neutrality.

pH Stability tests

The stability of the various modified zirconia particles were determined in three different ways. Firstly, the triazine dye-modified zirconia particles were treated with buffers of various pH and the ligand leakage was directly monitored from UV absorption of the supernatant. Secondly, radioactively labelled ligands were immobilised and the extent of leakage was detected from the release of radioactivity in the supernatant. Thirdly, the performance of the surface modified particles in HPLC column experiments were used as an indicator of ligand leakage.

(1) Direct monitoring of Cibacron Blue F3GA

trianzine dye ligand leakage in batch experiments: The Cibacron Blue F3GA modified zirconia particles were suspended in 100 mM sodium carbonate buffer solutions at different pH values and shaken for 24 h. After this time, the particles were centrifuged and the supernatant was examined for ligand leakage spectrophotometrically at 280 nm. In another experimental series, several different buffers were employed for pH stability tests of glucose derivatised zirconia modified with Cibacron Blue F3GA. The buffers used were sodium phosphate, sodium carbonate and β-alanine all at concentrations of 100 mM, as well as aqueous sodium hydroxide up to pH 13. The result of this experiment is shown in Fig. 8.

(2) Detection of leakage of ^{14}C labelled ligands in batch experiments: In a typical experiment 10 mg of modified zirconia particles were suspended in 2 ml of a 0.1 M sodium carbonate buffer and shaken for 24 h. After this time, two samples (each 0.5 ml) were taken and mixed with 4.5 ml scintillant liquid (Emulsifier-Safe purchased from Packard Instruments) and counted for 2 min. The particles were resuspended in a new buffer of increased pH. The pH was increased in steps of 0.5 and the whole procedure was repeated up to pH 14. The extent of ^{14}C ligand leakage is shown in Fig. 9.

(3) RP chromatographic performance as indicator of ligand leakage: For these experiments, the octadecyl modified zirconia was packed into columns (33 mm × 8 mm I.D.; column volume 1.66 ml). The HPLC equipment used consisted of two Waters pumps Model 6000 Å, a Waters gradient former Model 660, a Millipore Waters LC spectrometer Lambda Max Model 481, a Waters Data Module and a DuPont chart recorder. The testsample consisted of aniline, toluene and naphthalene (1 mg/ml each) and the mobile phase was water 0.1% TFA at a flow-rate of 1 ml/min. Analyte detection was performed at a wavelength of 254 nm.

The column was exposed to a 0.1 M carbonate buffer of pH 9.0 for 1000 column volumes at a flow-rate of 1.0 ml/min. After each 100 column volumes a performance test was carried out by injecting the test mixture. After 1000 column volumes the pH was increased by 0.5 units. A

decrease in retention time or a change in the plate number was used as an indicator of a decrease in ligand coverage.

The plate number was evaluated using the following expression:

$$N = 5.56\left(\frac{t_R}{t_{1/2}}\right)^2$$

where N = plate number, t_R = retention time and $t_{1/2}$ = bandwidth at half peak height.

To ensure that a change in retention time was due to a loss of ligands and not due to irreversibly adsorbed sample substances, the column was washed with 2-propanol at the end of each pH step and the sample was reinjected. No change or improvement in resolution was noted using this procedure.

Detection of "non-specific" protein interaction on carbohydrate and Glymo-derivatised zirconia sorbents

For different modified zirconia materials were tested, namely the zirconia particles modified with glucose, maltose and Glymo under either the anhydrous or the aqueous reaction conditions. The sorbents were packed into analytical stainless-steel columns (100 mm × 2 mm I.D.) and equilibrated in the chromatographic mobile phase. Three different mobile phases were used: 10 mM sodium carbonate buffer pH 6.5 with 0, 100 and 500 mM NaCl added. Three proteins were used as test solutes: bovine ribonuclease A (pI 8.9), bovine carbonic anhydrase (pI 5.9) and ovalbumin (pI 4.7). The experiments were run in triplicate at the different salt concentrations. The dead volume of the packed column was determined with acetone. The elution of these proteins were expressed in terms of elution volume of the protein divided by the elution volume of the acetone. Since a zirconia based material with 3000 Å pore size was used, there should be no exclusion effect and the proteins were expected to elute with an unchanged selectivity unless interactions between the protein and the particle surface occurs as a consequence of the pH instability of the sorbent or insufficient quality of the modification.

RESULTS AND DISCUSSION

A range of methods were used to determine the hydroxyl group density and the accessible naked zirconia surface area before and after chemical modification. A combination of these analytical methods, variations in the pretreatment of the particles and the chemical modification were all employed to optimise the modification procedure.

Determination of surface area

Phosphate anions are known to bind strongly to zirconia surfaces [16]. Therefore, the amount of bound phosphate ions on the support particles can be used to determine the surface area of the naked support or after chemical modification to determine the zirconol content of the remaining underivatised surface of the particles.

These measurements can be used as an alternative to elemental analysis to determine the success of the surface modification. The phosphate content determination with chemically modified zirconias has certain advantages over the traditional carbon content analysis. The assay can be readily carried out, is faster to use and is less expensive than the CH elemental analysis performed by a specialised laboratory. Due to the small specific area of macoporous zirconia particles, which result from the large pore size and a high specific gravity of the material, the experimental error by determining the carbon content of the modified zirconia also can be very significant (see Fig. 4).
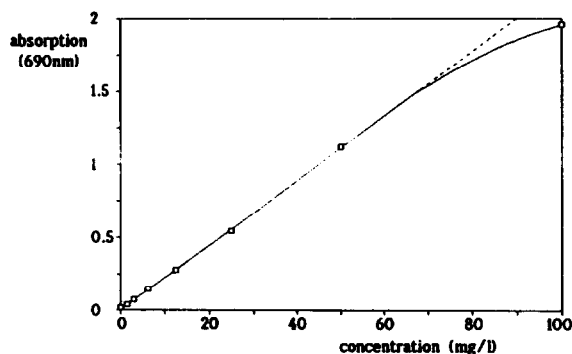

Fig. 4. Calibration curve of the phosphate molybdate complex monitored at 690 nm.

Table I shows the results of phosphate adsorption on different modified and unmodified zirconia support materials.

The results with the zirconias indicate that due to a heat treatment step in the manufacturing process the surface coverage by the immobilised ligands was low (≤ 2 μmol/m^2). In order to increase the hydroxyl group density to a level suitable for chemical modification of the surface, a pretreatment step to reintroduce the hydroxyl groups on the surface was performed. The zirconia particles were treated in an autoclave in a water steam atmosphere at 423 K for different times reaching from 1 to 16 h. After the treatment the particles were modified with a C$_{18}$-silane and the uncovered zirconia surface was determined by the adsorption of phosphate ions. The result of these experiments is presented in Fig. 5. The amount of phosphate able to bind to the ODS-modified zirconia surface reached its minimum after 6 h, indicating that a hydrothermal treatment of at least six hours of hydrothermal treatment is necessary to achieve maximum hydroxyl group density. Furthermore this result document that the hydrothermal treatment permits the reduction of the unmodified free surface area by 75%, thus leading to a much improved modification.

Determination of hydroxyl-group density on the zirconia surface

On a zirconia particle there are mainly two different sources of protons, the hydrogen of the
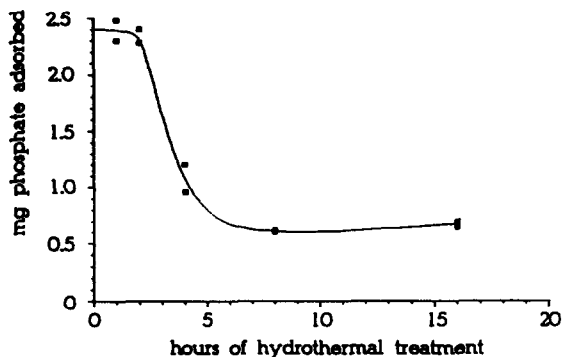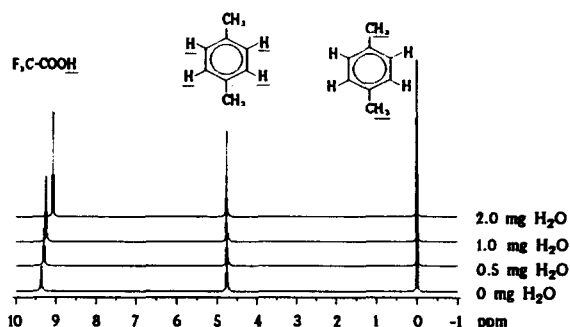
Fig. 6. ^{1}H NMR of a mixture between trifluoracetic acid and p-xylene with different contents of water.

hydroxyl groups associated with the surface zirconols and the hydrogens from the water adsorbed onto the particle. While the bulk water can be removed out of the pores and from the surface by heating the particles to 110°C, there will still be water molecules strongly adsorbed onto the surface which can not be removed by this procedure. Consequently, the method developed by Holík and Matějková [23] to determine the hydroxyl group density on silica support materials by suspending the particles in D-TFA was applied for the zirconia particles. Under these conditions deuterium exchanges very rapidly with the hydrogen of the water layer and the hydroxyl groups. The adsorbed water also dissolves in the TFA. Both effects have an influence on the resulting ^{1}H NMR spectrum (see Fig. 6). The amount of water dissolved in the acid will lead to a chemical shift of the signal while all protons (from the water and from the hydroxyl groups) will contribute to the signal intensity.

The amount of TFA (protonated form) was measured at 300 MHz by ^{1}H NMR. Fig. 7 shows the calibration curve for a different amount of water added to the solution of p-xylene in D-TFA. To evaluate the ^{1}H NMR signals both the chemical shift and the area of the TFA signal was determined relative to the chemical shift and the area of the methyl groups of p-xylene.

Chemical modification of zirconias

Two principally different methods for the modification of the surface of a sorbent particle

Fig. 5. Influence of different times of hydrothermal treatment on the surface coverage of the C$_{18}$ modification.
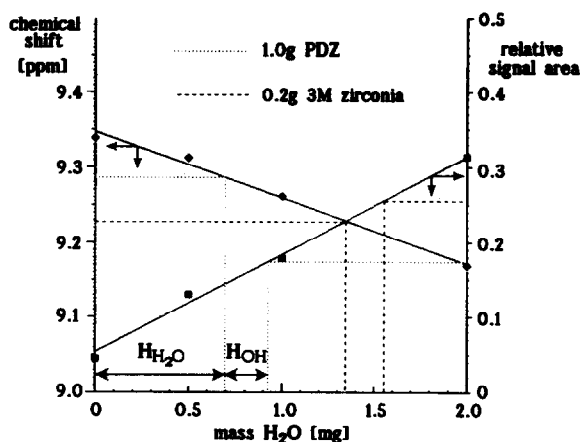
Fig. 7. Calibration of the ^{1}H NMR experiment to determine the hydroxyl group density of zirconia particles.

are available. The first approach is to use an organosilane which will react with a hydroxyl group present on the support surface. If a monofunctional organosilane is used, this will lead to a monomeric modification. The other possible route to introduce a desired interactive surface is to cover the surface of the particle with a polymer coating. This method was originally applied to silica particles to enhance the stability at high pH values.

Different procedures to modify zirconia were used to test a variety of chemical pathways for the introduction of desirable surface chemistries onto the particle surface. These different reactions result in either monomeric or polymeric modifications with different amounts of cross-linking. The derivatisation of zirconia with organosilanes can be performed under aqueous and non-aqueous conditions. One example of the reaction between an organosilane and the zirconia is the immobilisation of 3-mercaptopropyltriethoxysilane with subsequent attachment of Cibacron Blue F3GA. The modification of silica with Cibacron Blue F3GA is widely used to generate biomimetic affinity sorbents [26,32]. To study the derivatisation of zirconia particles a Cibacron Blue modification was chosen because it is easy to determine whether the modification was successful by observation of the intensity of colour of the final product, as well as permit easy monitoring of any ligand leakage.

The modification of zirconia with octadecyl-

dimethylchlorosilane in dry toluene produced a monomeric ligand with a single covalent bond between the silicon atom of the silane and the oxygen of the zirconol group. Like its silica based counterpart, this sorbent material exhibited strong hydrophobic properties.

Another possible route to produce reversed phase material is to attach a polymeric layer onto the surface [6,7]. Depending on the amount of polymer desired on the surface, different methods to prepare these sorbents are available. The polymeric layer should not be too thick otherwise it will fill up the pores and decrease the surface area to a very large extent. In the present investigations two levels of prepolymerised polybutadiene cross-linked with dicumylperoxide were employed.

In another approach, the zirconia particles were modified with carbohydrates. The purpose of these experiments was to produce a hydrophilic bonded phase which would be easily derivatised and which would have very low nonspecific interactions with proteins. Glucose and maltose were coupled to aminopropyl derivatized PDZ-zirconia, with a similar procedure which has been used for silica [27–29].

pH Stability tests in a batch experiment using dye-modified zirconia

To determine the chemical stability of the MPT-activated zirconia modified with Cibacron Blue F3GA, the dye sorbent was suspended in buffer solutions of various pH and then shaken for 24 h at room temperature. After this treatment the suspension was centrifuged and the supernatant was examined for dye leakage from the sorbent surface. The whole procedure was then repeated in a buffer adjusted at a pH 0.5 units higher than the previous experiment. Using these conditions no leakage was detected from pH 8.0 up to PH 9.5. At pH 10.0 the Cibacron Blue F3GA dye slowly leached from the surface, indicating that the MPT-modification is not stable under these conditions.

The supernatant was evaporated and the residue remaining was used for elemental analysis. The material was tested for its nitrogen, silicon and zirconium content. According to the pre-

sumed structure, the cleavage could occur at three different places: (1) the modified zirconia bead was actually dissolving, which would give positive results for the zirconium, silicon and nitrogen content, (2) the cleavage occurred between the zirconol oxygen atom and the silicon atom, giving positive results for the silicon and nitrogen 'content but negative results for the zirconium, and (3) the cleavage occurred at the sulphur atom between the mercaptopropyl group and the dye molecule, resulting in very small amounts of both silicon and zirconium.

The result of the elemental analysis was 8.8% nitrogen, 1.3% silicon and 0.0047% zirconium, indicating that the cleavage at pH 10.0 with the MPT-activated dye sorbent had predominately occurred at the Zr–O–Si bond.

pH Stability tests using the carbohydrate-dye-modified zirconia

It has been reported [33] that some ions, such as phosphate and carbonate, are able to displace covalently linked organosilanes from alumina. To investigate, whether this effect also occurs the case with zirconia a stability test was performed using different buffers: namely 0.1 M phosphate, carbonate and β-alanine buffers. Water titrated with NaOH was also used as a reference. The stability tests were performed with the glucose-Cibacron Blue F3GA-modified zirconia in batch experiments. Evidence for a loss of the modification was monitored at 280 nm. In each case 450 mg of these dye–zirconia particles were suspended in 5 ml buffer and shaken for 24 h each. The experiments were started at pH 9.0 and the pH was increased by 1 unit after each run. The results of the experiment, carried out in triplicate, are presented in Table II.

This experiment showed that there were no significant differences in stability of this bonded phase in the different buffer solutions, indicating that these ions are not able in this case to diplace covalently attached organosilanes from the zirconia surface. The surface modification showed high stability, at least up to pH 11.0.

The modified zirconia produced with immobilised maltose were stable up to pH 12 as docu-

TABLE II

INFLUENCE OF DIFFERENT BUFFERS ON THE STABILITY OF CARBOHYDRATE-CIBACRON BLUE-MODIFIED ZIRCONIA

The values are given in absorbance units at 280 nm.

Buffer	pH				
	9.0	10.0	11.0	12.0	13.0
Phosphate	0.26	0.25	0.25	0.31	0.46
β-Alanine	0.22	0.25	0.33	0.38	0.56
Carbonate	0.25	0.32	0.34	0.36	0.56
NaOH	0.31	0.26	0.28	0.46	0.59

mented in Table III. In this experimental series, two materials were compared, one with and one without butadienediepoxide cross-linkage. Both materials were derivatised with Cibacron Blue F3GA. Stability tests were carried out with a 100 mM phosphate buffer. These results (Table III and Fig. 8) show clearly that not only the cross-linked sorbent but also the non-cross-linked sorbent are remarkably stable. The high stability of these modified zirconia sorbents was also seen when the glucose-dye-modified zirconia particles were suspended in 1 M sodium hydroxide and treated for 24 h. After washing to neutrality and drying no leakage of the dye could be detected. These particles clearly show a substantially higher pH stability compared to silica particles.

The results from the "non-specific" protein interaction measurements are presented in Table IVa–c. As the results for both the glucose and maltose modified zirconias were very similar,
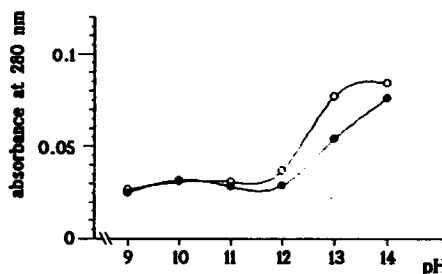

Fig. 8. pH stability test using the maltose modified zirconia in its cross-linked (●) and non-cross-linked (○) form.

TABLE III

LIGAND LEAKAGE OF CARBOHYDRATE-CIBACRON BLUE-MODIFIED ZIRCONIA

Support	pH					
	9.0	10.0	11.0	12.0	13.0	14.0
Non-cross-linked	0.027	0.031	0.031	0.037	0.077	0.081
Cross-linked	0.025	0.032	0.029	0.029	0.054	0.076

only the results for the glucose modified particles are listed. The different modified hydrophilic sorbents showed distinctly different properties. The Glymo sorbent prepared by immobilising the Glymo-silane in water at an acidic pH has a polymeric coating, which is covalently attached to the surface. This coating results in a good coverage of the surface as indicated by the protein elution characteristics of the sorbent. However, this kind of modification leads to a thick polymer layer, reducing the chromatographic performance of the support due to increased pore diffusion effects. Both the carbohydrate-modified particles and the support synthesised with the Glymo-silane under anhydrous conditions result in a monomeric modification with a controlled thickness of the interactive surface.

As evident from the experimental data obtained with these monomeric modified supports, the zirconia particles with the carbohydrate ligands showed superior performance over the particles modified with the Glymo-silane. This difference might be explained by the size of the carbohydrate ligand compared to the Glymo-group, thereby preventing the protein from reaching the zirconia surface. There is also a qualitative difference between these two materials. While the carbohydrate modified sorbent interacted only with the most basic protein (ribonuclease A), the Glymo-modified particles also adsorbed the more acidic proteins ovalbumin and carbonic anhydrase. This result indicates, that both the anionic and cationic groups, e.g. Lewis/Brønsted acid/base groups, present on the zirconia surface can interact with proteins when some types of chemical modifications are used.

TABLE IV

PROTEIN INTERACTION ON DIFFERENT HYDROPHILIC MODIFIED ZIRCONIA SUPPORTS

(a) Glucose-modified particles, (b) Glymo modified under anhydrous conditions, (c) Glymo modified under acidic aqueous conditions. The elution of the proteins is expressed in elution volume of the protein divided by the elution volume of acetone.

	Protein	NaCl concentration (mM)		
		0	100	500
a	Ovalbumin	0.97	1.05	1.18
	Carbonic anhydrase	1.07	1.07	1.05
	Ribonuclease A	Not eluted	2.01	1.04
b	Ovalbumin	Not eluted	1.09	0.99
	Carbonic anhydrase	Not eluted	Not eluted	1.32
	Ribonuclease A	Not eluted	1.74	1.20
c	Ovalbumin	0.96	1.02	1.07
	Carbonic anhydrase	0.99	1.01	1.04
	Ribonuclease A	Not eluted	1.27	1.13
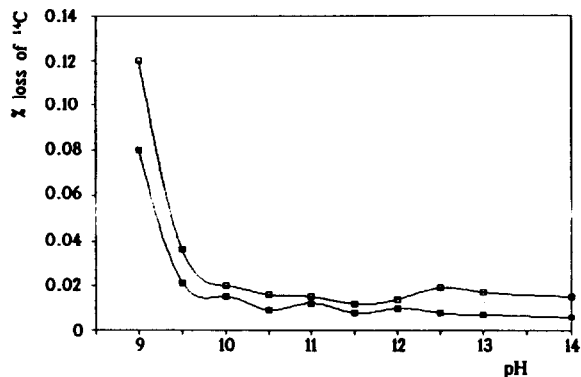
Fig. 9. pH stability test of the cross-linked (■) and the non-cross-linked (□) [^{14}C]glucose.

Fig. 10. pH stability tests of C$_{18}$-modified 3M zirconia. pH 9.0 (□), pH 10.0 (■), pH 11.0 (◇), pH 12.0 (◆), pH 13.0 (△).

Stability test of porous zirconia, hydrophilically modified with ^{14}C labeled glucose

The results of the stability tests of porous zirconia modified with ^{14}C-labelled glucose are shown in Fig. 9. As expected, the cross-linked sorbent is slightly more stable than the sorbent that was not cross-linked. The apparent leakage of a small amount of [^{14}C]glucose of both materials at pH 9.0 and 9.5 might be a consequence of some glucose molecules not being covalently attached to the surface but physically adsorbed, and washed off in the early stages of the experiment.

Stability test of the octadecyl-modified zirconia

The idea behind using a reversed phase modified sorbent in separation experiments to determine the pH stability was the assumption that a loss in ligands would uncover the hydrophilic surface of the zirconia particles. This decrease in hydrophobicity would therefore impair the reversed-phase performance of the support, i.e. any loss of ligands should result in significant changes of the retention time. Furthermore, the presents of uncovered zirconia surface should lead to a broadening of the elution peaks due to secondary equilibria associated with interactions other than hydrophobic in nature. The results of the pH stability tests using reversed-phase chromatographic performance as indicator for changes in the surface modification of the sorbents are shown in Fig. 10. As evident, throughout these experiments no change in performance could be observed, indicating that the ODS-
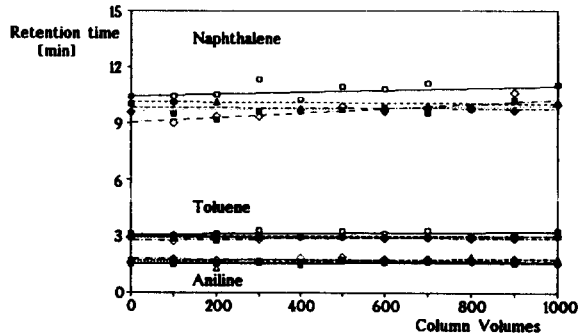
modification is able to withstand at least 1000 column volumes at pH 13.0 without affecting resolution. To ensure that the separation is due to a reversed-phase mechanism, the experiment was repeated using the non-modified zirconia where a totally different retention behaviour was observed. Fig. 11a shows the chromatogram at the beginning of the experiment, Fig. 11b exhibits the chromatogram at the end of the test series after 1000 column volumes at pH 13.0 whilst in Fig. 11c the result with the non-modified packing is shown.
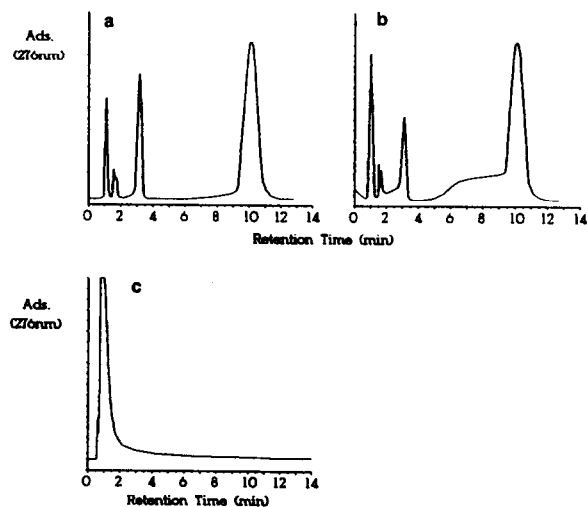

Fig. 11. Chromatograms on C$_{18}$-modified zirconia. (a) Chromatogram at the beginning of the experiment; (b) chromatogram after washing with 1000 column volumes at pH 13; (c) chromatogram on a non-modified support.

CONCLUSIONS

The results of the experiments presented in the paper document methods to produce zirconia-based chromatographic sorbent materials with superior chemical stability compared to silica-based sorbents and better physical characteristics than sorbents based on organic polymers. Analytical techniques were developed to permit characterisation of these zirconia-based sorbents. In further studies the suitability of other types of chemical modifications, e.g. the application of zirconia-based sorbents in affinity chromatography will be described. The results gained so far indicate that the modification procedures using organosilanes to introduce active groups onto the zirconia surface may be universally applicable.

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