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Review

Controlled-pore glass as a stationary phase in chromatography

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ABSTRACT

Controlled-pore glass is well known as a stationary phase in chromatography and is used mainly for size-exclusion and adsorption chromatography. Owing to its strong adsorption properties, especially for charged molecules such as proteins, its applicability originally was limited. Nowadays modified controlled-pore glass is available with either an extremely low adsorptivity or with bonded reactive groups, allowing further reactions including biologically specific ligands. Hence the material is used also in affinity and ion-exchange chromatography. In addition to a variable surface chemistry, the pore-size distribution is extremely sharp and can be adapted in a range between 7.5 and 400 nm pore diameter at high pore volumes up to 70%. Both properties are complemented by high strength and hence structural stability, chemical stability and defined chemical composition. Controllable production conditions allow the application of a well characterized material which is used in preparative- and production-scale chromatography. The main applications are in the separation of macromolecules, mainly biologically active compounds.

CONTENTS

1. INTRODUCTION

In 1965, Haller [1] published a paper that started the revival of controlled-pore glass. Originally, porous glass was developed in order to overcome technical difficulties in melting quartz glass. Owing to the lack of suitable refractory material, the high melting temperature of quartz was prohibitive. Hood and Nordberg [2–4] had

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developed in the 1930s a process based on a glass composition exhibiting a miscibility gap. The melting temperature of such glasses was less than 1500°C. After phase separation, the glass was leached by an acid treatment and sintered at about 1200°C. This glass is usually referred to as Vycor. The intermediate form is the so-called Vycor 7930. The void volume of this glass is about 28% with an average pore diameter of 4 nm [5]. Haller [1] prepared for chromatographic applications a series of porous glasses with pore diameters between 17 and 170 nm and a pore volume of about 50%. The grain size fraction was 150–300 μm (50–100 mesh). The main advantage of this material was the extremely narrow pore-size distribution; 95% of the pore size was within ±20% of the average pore size. The applications were mainly in the separation of macromolecular substances, cell components and virus particles, *e.g.*, tobacco mosaic virus from tobacco ring spot virus or albumin from southern bean mosaic virus. Some features, such as the rigidity of the chromatographic bed, the chemical inertness, the low flow resistance, the ability of the columns to withstand heat sterilization and cleaning with hot nitric acid, were new for a chromatographic material at that time and started tremendous activity in testing controlled-pore glass.

The main databases for this review were *Chemical Abstracts* and BIOSIS, covering the literature from 1968 and 1969, respectively. From about 180 references dealing with the application of controlled-pore glass in liquid chromatography, we have selected those which we consider significant.

2. PRODUCTION

Since the first patents, the main production steps for porous glass have remained more or less unchanged. The glass is produced from a ternary system consisting of silica (50–75%), sodium oxide (1–10%) and boric acid (to 100%). For special purposes, *e.g.*, with higher hydrolytic stability or larger pore sizes, substances such as alumina or lime can be added. The homogeneous molten glass is phase separated by heat treatment between 500 and 750°C. The heat treatment should, according to Haller [6], conform to the equation $r^n = kte^{-m/T}$, where r is the desired pore radius, t is the time in hours, T is the temperature and m, n and k are experimentally determined constants. The phase-separated glass is then crushed and screened, resulting in a glass powder of the desired grain dimensions. The heterogeneous phases of the demixed glass are completely interconnected and one of the phases, consisting mainly of a mixture of sodium borate and boric oxide, is leached by mineral acid or water at elevated temperature. After the acid treatment, and depending on the temperature of the phase separation, the pore volume is still low, the pores being filled with colloidal silica. In order to attain a higher pore volume, a second treatment using sodium hydroxide leads to porous glass granules with pore sizes up to 400 nm and pore volumes exceeding 70%. An extensive treatment of this glass system was given by Janowski and Heyer [7].

Usually the glass is available as an irregularly shaped packing material. For special purposes it might make sense to use the material as spherical particles. Such spherical particles can be produced, according to Hammel and Allersma [8], by passing the irregularly shaped particles through an inclined rotating tube furnace in which the centre portion is at a temperature of about 925–1100°C. Such porous glass beads for chromatography were also reported by Greiner-Bär and Schäfer [9].

Hammel and Allersma [8] also claimed the use of glass compositions with a silica

content of less than 50% in order to increase the crush strength and to obtain large pore volumes without any caustic treatment. Such a production requires a long time for the acid leaching. Even with extended times some colloidal silica remains in the pores and will influence the performance in the chromatographic column.

3. MATERIAL PROPERTIES

As suggested by the designation controlled-pore glass, the main feature of porous glass is the very sharp pore-size distribution in the range between a few nm and about 400 nm. Typical pore-distributions for porous glass were given by Schnabel and Langer [10] (Fig. 1).

Different in composition and in the resulting pore sizes is the so-called Shirasu porous glass [11]. The pore sizes of such glasses are in the range 200–10000 nm.

The pore characteristics can be determined either by the analysis of the hysteresis in physical adsorption, commonly using nitrogen adsorption, or by mercury penetration. For a limited number of cases small-angle X-ray scattering can also be used [12]. Physical adsorption is limited to pore sizes below 30 nm, depending on the resolution of the equipment. The sample is cooled to the temperature of liquid nitrogen (77.4 K) and the adsorption and desorption isotherms are recorded. They yield information on specific surface area and pore volume in addition to pore diameter and shape. In mercury porosimetry the non-wetting properties of mercury are used for the determination of pore size and pore-size distribution. The pressure needed to force mercury into a pore is related to the diameter of the pore. Instruments with a maximum pressure of about 4000 bar (corresponding to a minimum pore diameter of 4 nm) are commercially available.

Nowadays materials can be made with reproducible pore-size distributions where 95% of the pores are within $\pm 5\%$ of the average value, as was demonstrated by Langer *et al.* [13] (Fig. 2).

In Table 1 the properties of porous Vycor 7930 according to Kleinteich [5] are listed.

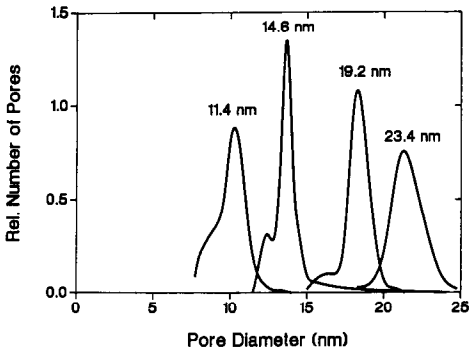

Fig. 1. Typical pore-size distributions of four different BIORAN porous glasses, measured by mercury porosimetry. Values on the curves are the pore diameters corresponding to the 90% value of the cumulated distribution function.

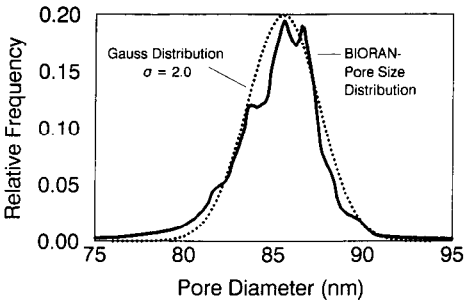

Fig. 2. Typical pore-size distribution of BIORAN porous glass (solid line), measured by mercury porosimetry, with a Gauss normal distribution fit (dotted line). With a mean pore diameter of 85.5 nm, 95% of pores are between 81.6 and 89.4 nm in diameter.

TABLE 1

PROPERTIES OF POROUS VYCOR 7930 [5]

Property	Value
Apparent density (dry)	1.45 g/cm^3
Porosity	28 vol.%
Water capacity	25 wt.%
Average pore diameter	4 nm
Specific surface area	150–200 m^2/g
Silica content	*ca.* 96%
Temperature limit	650°C

Today controlled-pore glasses for chromatography are available with different specifications. Table 2 lists general limits according to different suppliers.

The surface of glass contains reactive silanol groups. The amount is about 4.7/nm^2, corresponding to 8 μmol/m^2 [10]. Practical values for bonded silanes are of

TABLE 2

GENERAL DATA FOR CONTROLLED-PORE GLASSES

BIORAN-CPG: Schott Glaswerke BioTech, Mainz, Germany. CPG: CPG, Fairfield, NJ, U.S.A.

Property	BIORAN-CPG	CPG
Pore size	30–400 nm	7.5–300 nm
Specific pore volume	0.5–1.2 cm^3/g	0.4–0.8 cm^3/g
Specific surface area	10–300 m^2/g	7–340 m^2/g
Particle size	30–250 μm	37–177 μm
Surface modification	Silanol	Silanol
	Diol	Glyceryl (Diol)
	Amino	Aminopropyl, aminoaryl
	Chloropropyl	Diethylaminoethyl (DEAE)
	Octyl	Carboxymethyl
	Octadecyl	Carboxyl

the order of 3 μmol/m^2. If trifunctional compounds of the type $RSiX_3$ are taken for the reaction, this value is the same order as the binding capacity of silica. In this instance three-dimensionally linked products are obtained [14]. An extensive treatment of silylated surfaces was given by Leyden and Collins [15].

4. APPLICATIONS IN CHROMATOGRAPHY

As early as 1963, Jaschin *et al.* [16] demonstrated the separation of polar and non-polar compounds using porous glass in gas chromatography. Although since then numerous papers have been published on the applicability of porous glass in gas chromatography, the main field for controlled-pore glass remains in liquid chromatography, where full advantage can be taken of the material properties.

The discussion as to whether spherical particles or irregularly shaped material should be used as a stationary phase is still unsettled. It is claimed that the column lifetime using spherical particles is superior to that of columns packed with irregularly shaped particles owing to the lower fines content. On the other hand, Laird *et al.* [17] stated, based on plate-height studies, that "little if any advantage arises from the use of spherical particles rather than broken chips". As already mentioned, controlled-pore glass can be produced in both shapes. Nevertheless, most of the literature deals with irregularly shaped particles. Despite the fact that the material is offered with grain sizes larger than 30 μm (see Table 2), the material can be crushed and screened by sedimentation in the range 5–10 μm for analytical purposes.

4.1. Size-exclusion chromatography

Initial applications of controlled-pore glass in liquid chromatography were for gel permeation chromatography (GPC), also referred to as size-exclusion chromatography (SEC) [1]. Because of the extremely sharp pore-size distribution and structural stability, porous glass is an appropriate material for the separation of molecules by molecular size or molecular weight.

The main applications concern proteins [18–22], human and animal viruses [1,23–28], dextrans [29,30], lipid suspensions [31,32], pigments [33], polymers [34,35] and micelle sizes [36,37].

The main obstacle in using controlled-pore glass in size-exclusion chromatography is the activity of the glass surface, leading to unwanted adsorption. A breakthrough was the development of methods for the tailored reduction of active surface sites. At the end of the 1960s, Sebestian and Halász [38,39] developed a process for the chemical silanization of the surfaces of inorganic solid bodies resulting in a chemically modified stationary phase component for chromatography known as "Halász brushes".

Mizutani [21] used a siliconized porous glass for the separation of a mixture of albumin and hemoglobin. Depending on the buffer, a recovery of 32% for albumin was obtained while hemoglobin was adsorbed on the column. The separation of bovine globulin in 0.01 *M* phosphate buffer at pH 7.4 showed that α_2-globulin was eluted and β- and γ-globulin were adsorbed by hydrophobic interactions. It was demonstrated that at high salt concentrations the coated glass adsorbs significant amounts of protein by hydrophobic bonding.

One might argue whether the mechanism involves adsorption or size-exclusion.

It could be also a type of liquid–liquid partition process with secondary effects arising from adsorption. It seems very likely that in all instances coupled mechanisms are responsible for the separation.

Mori and Kato [22] studied the effects of increasing phosphate buffer or neutral electrolyte concentrations in the mobile phase on the retention volume of proteins using diol-bonded porous glasses. Size-exclusion separation was achieved with a mobile phase containing 0.1 *M* phosphate and 0.1 *M* sodium chloride at pH 7.0.

The preparation of immunoglobulin concentrates was described by Haller *et al.* [18]. With this method, using porous glass with a pore size of 17.5 nm, human serum was fractionated. Immunoglobulin (Ig) M, α_2-macroglobulin and some immunoglobulin G were in the exclusion peak. Later the method was also applied to human plasma. With 0.02 *M* Tris, 0.02 *M* sodium citrate, 1 *M* sodium chloride at a pH between 8 and 9 as eluent, fibrinogen and lipids eluted in the void volume. Reducing the ionic strength of the eluent to 0.1 *M*, fibrinogen was adsorbed.

Size-exclusion chromatography is considered to be one of the most efficient and simple ways of purifying virus materials. Krasilnikov *et al.* [27] used Tris-modified porous glass [tris(hydroxymethyl)aminomethane followed by reduction with sodium borohydride] with an average pore diameter of 110 nm and a porosity of 1.6 cm^3/g for the purification of tick-borne encephalitis (TBE) virus suspension. The procedure allowed over 99% of ballast proteins to be removed.

A general equation for the selection of pore size in size-exclusion chromatography was given by Haller *et al.* [30]. Porous glasses with particle sizes between 75 and 125 μm (120–200 mesh) and five pore sizes from 8.4 to 51.7 nm were used. Evolving from relatively simple assumptions, an equation relating pore size, molecular weight and elution coefficient of specially prepared highly monodisperse dextran was developed.

4.2. Adsorption chromatography

In principle, adsorption effects occur also in other chromatographic methods. Adsorption chromatography is mainly applied for the isolation of biologically active substances whereas SEC is used for the concentration and purification of biological material and other high-molecular-weight compounds. In this paper we shall follow categorization according to the authors of the literature cited.

Adsorption chromatography with controlled pore glass has been applied to proteins [40–47], interferons [48–52] and viruses [53] and to special biologically derived substances such as erythropoietin [54], interleukin-2 [55], factor VIII [56], human growth factor [57], procollagen [58], tumour necrosis factor [59] and nucleic acids [60]. It should be mentioned that also the isolation of other biological substances is included in these references. Mizutani [46] reviewed the adsorption properties and isolation possibilities using controlled-pore glass for substances such as macrophages, blood platelets, amino acids, nucleosides and nucleotides, glucosamine, serum albumin, lysozyme, α-globulin, γ-globulin, IgG, IgM, peroxidase, glycine, hemoglobin, catalase, chymotrypsinogen A, malate dehydrogenase, aldolase, cytochrome *c*, myoglobin, hydroxybutyrate apodehydrogenase, influenza virus, collagenase, interferon, chlorophyll–protein complex, protein kinase and insulin.

Mizutani [46] also gave a comparison between a siliconized porous glass and native porous glass. The amounts of insulin, atropine sulphate, physostigamine

salicylate and diazepam adsorbed were higher on the siliconized surfaces than on the native surface. For the substances examined the siliconized surface adsorbs more drugs than the native surface in isotonic solution. On the other hand, the protein adsorption on a diol-modified porous glass was about 9% of that on unmodified glass. The reported value was 0.14 mg/m^2. Langer and Schnabel [47] found a bovine serum albumin adsorption of 2 μg/m^2 on a diol-modified porous glass. They also gave a mathematical treatment of adsorption on porous bodies, describing the main features of the experimentally determined data.

The comparatively low adsorption value for BSA may be due to a more efficient surface modification in term of blocking active silanol groups, following the suggestion of Mizutani [46] that silanol groups in contrast to alcoholic OH on glass surfaces are essential for protein adsorption. On the other hand, other parameters such as concentration, specific surface area, particle size and pore size play an important part in the adsorption behaviour, as was demonstrated by Langer and Schnabel [47]. Mizutani [46] concluded from comparisons of the adsorption order of standard proteins with similar molecular weights that other factors such as the helix content or the aggregative forces in aqueous solution might also have an influence on the adsorption of proteins.

Sulkowski [45] studied the elution efficiency of different substituted ammonium chlorides, in particular for lysozyme. He found an almost complete restoration of controlled-pore glass using tetramethylammonium chloride (TMAC). In addition, it was recommended to try a combination of sodium chloride and ethylene glycol and TMAC when each elution mode on its own is inadequate.

Bock *et al.* [40] described the application of controlled-pore glass to the apoenzyme form of D-(−)-β-hydroxybutyrate dehydrogenase (BDH) using a chaotropic buffer in the elution scheme. The specific activity was approximately 100-fold greater than that of phospholipase A digestion. Dithiothreitol was added to the buffers in order to stabilize the apoBDH. The presence of the chaotropic salt lithium bromide in the extraction medium minimized the aggregation of the mitochondrial membrane-bound protein.

Chaotropic agents, in particular potassium thiocyanate, have also been applied for the elution of human leukozyte interferon [HuIFN-α(Le)]. With TMAC as the eluting agent, a purification factor of 21-fold with a recovery of 91% of interferon can be achieved [50].

Concentration and purification steps for interferon are performed using controlled-pore glass with recoveries exceeding 90%, and supplementary steps such as gel filtration and chromatography with phenyl-Sepharose [49], zinc chelate [48], antibody affinity chromatography [51], ultrafiltration with size-exclusion high-performance liquid chromatography [50] or blue-Sepharose chromatography can also be applied.

Further isolation methods leading to purified biologically active substances of practical interest have been described in detail [53–60].

4.3. Affinity chromatography

The most powerful technique for the purification of biological substances and one of the most challenging tasks for a biochemist is affinity chromatography. Whereas in all of the other chromatographic techniques more or less non-specific

interactions are responsible for the separation, affinity chromatography uses extremely specific interactions to pick a single substance out of a complex mixture. On the one hand this leads to simple single-step purification procedures, but on the other suitable ligands must be found that can be covalently linked to the matrix and that form reversible complexes with the substance to be purified.

Porous glasses can be silanized and functional groups can be introduced [38]. Further general reactions for coupling ligands have been described in detail [61].

Because of the extreme selectivity a wide variety of ligands can be used. Some of the main substance classes are proteins (enzymes), antigens and antibodies, mono- and polysaccharides, nucleic acids and nucleosides, lectins, reactive dyes and metal chelates. Some examples may demonstrate the applicability of controlled-pore glass in affinity chromatography.

A one-step isolation of polyclonal IgA or IgM is obtained with covalently bound anti-IgA or anti-IgM antibodies [62]. The porous glass was reacted with triethoxysilylpropylamine and activated with glutaraldehyde. After elution of IgA or IgM with propionic acid, a purification factor of 15 with 15% recovery for IgA and a purification factor of 38 with 42% recovery for IgM demonstrated the fast and efficient process. Even after more than 20 adsorption–elution cycles the capacity of the columns was not reduced.

Radioimmunoassays by coupling highly specific antibodies on a porous glass were used for measuring serum or plasma digoxin levels [63]. Again the antibody stability was excellent over several months at room temperature.

A different application is the use of controlled-pore glass-8-hydroxyquinoline for the removal of trace metals for preparing nutrient solution cultures [64]. The trace metals can be quantitatively recovered by elution with 1.2 *N* hydrochloric acid.

Lobarzewski and co-workers [65,66] used vanillin-activated amino-modified controlled-pore glass for the separation of anionic proteins. The separated anionic protein fractions contained peroxidase, laccase and glucose oxidase.

Amino-modified controlled-pore glass activated by carbodiimide and linked to polygalacturonic acid (PG) was used for the purification of pectinases [67]. A two times better affinity volume relating to PG activity in comparison with amino-modified silica gel was obtained.

5. CONCLUSIONS

In the history of controlled-pore glass it has been demonstrated that the main properties, in particular the extremely narrow pore sizes, the rigidity of the chromatographic bed, the chemical stability, the high pore volume and the surface chemistry are of benefit for the concentration, separation and isolation of macromolecular substances, especially biologically active compounds. The active glass surface originally seemed to be a limitation owing to the irreversible adsorption of many proteins. This was subsequently converted into a usable property by the introduction of methods for the silanization and thus functionalization of the surface silanol groups [38].

Chang *et al.* [68] prepared a "glycophase" controlled-pore glass support by polymerizing triglycidylglycerol on a glycerylpropylsilyl bonded phase. Using this support, coatings of oxirane monomers, diethylaminoethyl (DEAE), methyldiamino-

ethyl (QAE), carboxymethyl (CM) and sulphonylpropyl (SP) were also applied, leading to ion-exchange properties. Such phases combine the advantages of porous glass with protein-adapted surfaces.

Most of the applications in chromatography relate to the preparative and industrial scales rather than to analytical chromatography. After years with less successful alternatives, Margolis and co-workers [56,69] developed a simple process for the large-scale manufacture of "high-purity" factor VIII. A crude concentrate of the cryoprecipitate is treated with controlled-pore glass [50 nm pore diameter, 75–125 μm (120–200 mesh)] in proportion of 20–30 ml of glass to 1 g input of protein. The loss of factor VIII fraction at this stage was less than 10%. The authors claim, that the developed method is flexible enough to be scaled up, according to the demand.

Nowadays, using surface-modified porous glass membranes in combination with controlled-pore glass chromatography, it should be possible to devise simple integrated processes using the same material in all steps and thus diminishing any contamination of the product.

Some new aspects of the variety of porous glasses have been described recently [70]. With the increase in biotechnological processes and with the need for highly purified biologically active material, controlled-pore glass might acquire a key position in both.

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