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COMPARISON OF GELS USED FOR MOLECULAR SIEVING OF PROTEINS BY ELECTRON MICROSCOPY AND PORE PARAMETERS DETERMINATIONS

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SUMMARY

A comparative ultrastructural study has been performed on three series of gels: Ultrogel (polyacrylamide–agarose), Sephadex G (crosslinked dextran) and Bio-Gel P (polyacrylamide). Scanning electron microscopy, and measurements of specific area, pore volume and pore size distribution have been carried out in the dry state. Correlations with certain known properties of these gels in the hydrated state are made.

INTRODUCTION

The use of natural or synthetic macromolecular matrices for the chromatographic separation of biological compounds in the aqueous phase is a technique first developed about 15 years ago. The porosity of these supports, which are insoluble but swell in water, is utilized for the separation of molecules as a function of their molecular weights (and thus approximately of their volumes) by simple elution from a column[1]. Porath and Flodin[2,3] were the first to develop this principle, by using the natural polysaccharide dextran crosslinked by a bifunctional reagent such as epichlorohydrin in a basic medium[4]. Another polysaccharide, agarose, was recommended several years later by Hjertén[5]. The hydrogen bonds which stabilize this gel give it a rigidity greater than that of dextran gels. Hjertén also developed a synthetic, polymerized, acrylamide-based gel, crosslinked by a bis-acrylamide[6]. Various degrees of polymer porosity could be obtained by varying the concentration of the crosslinking agent.

In 1971, Uriel *et al.*[7] began to develop a gel which combined the properties of synthetic acrylamide polymers and those of natural polymers, particularly the rigidity of agarose. These mixed gels are now known as Ultrogels AcA. It was reported[8,9] that the performance of these polyacrylamide–agarose gels was greater than that of other gels normally used for protein separations, especially in the molecular weight range 50,000–1,200,000 daltons. The superiority of these mixed gels is shown not only by a

greater mechanical resistance but also by a greater separation efficiency. Thus, Ultrogels have a number of theoretical plates per metre which is much greater than that of other gels for similar flow-rates[10].

The purpose of the present work was to determine the differences between dextran (Sephadex G), polyacrylamide (Bio-Gels P) and mixed polyacrylamide–agarose gels by employing such conventional physical techniques as scanning electron microscopy and porosity determinations. We wished to obtain further insight into the relationship between the physical properties of a gel and its filtration properties.

Among the voluminous literature concerning the use of hydrophilic gels for protein filtration, there are very few references to the physical properties of such gels. This is especially true for the microscopic observation of their internal and external structures. De Mets and Lagasse[11,12] were the first to publish photomicrographs of Sephadex and Bio-Gel beads. The specific surface area and the porosity of a chromatographic support are important parameters[13]. In the case of gels which are swelled in an aqueous medium, these parameters are less representative. Volkova *et al.*[14] showed that for poly(hydroxyethyl methacrylate) gels the specific area increased as the exclusion limit was augmented.

MATERIALS AND METHODS

Reagents

Sephadex G-75, G-150 and G-200 were purchased from Pharmacia (Le Chesnay, France), Bio-Gel P-60, P-100 and P-300 from Touzart and Matignon (Paris, France) and Ultrogels AcA-54, AcA-44, Aca-34 and AcA-22 from IBF-Réactifs, Pharmindustrie (Clichy, France). All of the other reagents were of high purity and commercially available.

Dehydration of Ultrogels AcA

The four types of Ultrogel AcA were dried by a progressive dehydration according to a method analogous to that described by Gribnau *et al.*[15]. All the gels were dried under identical conditions, using 100-ml samples of gel in suspension which were first mixed with an equal volume of deionized water. The sample was placed in a sintered glass büchner funnel and agitated with a propeller. The output of the funnel was regulated to obtain a flow-rate of 50 ml/h. The liquid level in the funnel was maintained constant by the continuous addition of 1 l of absolute ethanol. A water–ethanol gradient was thus obtained, water being completely replaced by the alcohol at the end of the operation. A second gradient was prepared under identical conditions, but with diethyl ether instead of ethanol. The Ultrogels were washed on the same sintered glass with 1 to 3 volumes of diethyl ether, drying *in vacuo* between each washing. Finally, the gels were air-dried for several hours and then placed in desiccators *in vacuo*. The gels were stored in stoppered bottles. Sephadex G and Bio-Gel P gels were obtained dry and were used directly.

Scanning electron microscopy

Dehydrated gel samples were first glued to the sample holder with silver glue and then covered with a double carbon–gold film by evaporation *in vacuo*. The gels thus become conductors to the electron beam. The internal structure of the beads was

visualized by first grinding them after chilling in liquid nitrogen. They were then treated as above. The images obtained are given by the secondary electrons of the gold layer. A JEOL 100-C electron microscope was used with an acceleration voltage of 40 kV in order to obtain maximal resolving power, *i.e.*, 50 Å.

Determination of specific area

BET method was used on a nitrogen point in a Ströhlein apparatus. Before the determination, each sample was completely degassed by passage of nitrogen at 50–60° for 5–35 h.

Determination of porosity

The technique utilized involved the penetration of mercury under pressure in a Carlo-Erba 70-H apparatus. Measurements were performed after degassing the samples for 2 h *in vacuo*. The measurement domain is located in a pore radius included between 40 Å and 6.6 μm, corresponding to a maximal pressure of 200 MPa.

RESULTS

Comparative scanning electron microscopy

The exterior appearance of the beads (Figs. 1–3) is such that we may conclude that each of the three types of gels are perfectly spherical with quite similar structures. The surface of Sephadex G (Fig. 1) is highly folded, in agreement with previous observations[11,12,15]. In contrast, the surface of Bio-Gels (Fig. 2), even that of the least crosslinked, P-100, is very smooth and homogeneous. Ultrogels AcA (Fig. 3) have a smooth surface, similar to that of the Bio-Gels P, which is comparable for the series of four gels of differing degrees of crosslinking.

The most interesting differences involve the internal structures of the gels (Figs. 1–3). Sephadex G gels, and especially the least crosslinked gel, present a smooth and dense mass within which are located craters, whose size and number increase from the periphery towards the centre (Fig. 1b). The superficial alveolar structure,

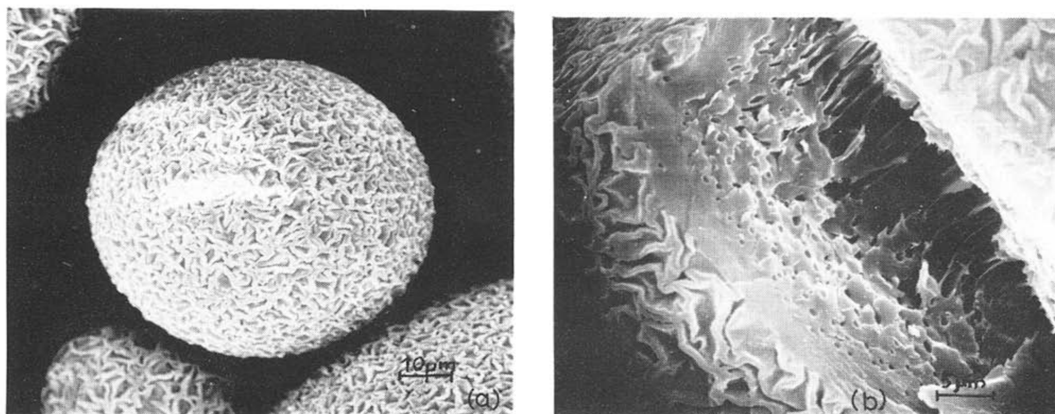

Fig. 1. Scanning electron photomicrograph of Sephadex G-150 beads. a = External aspect; b = internal aspect after grinding the gel in liquid nitrogen.

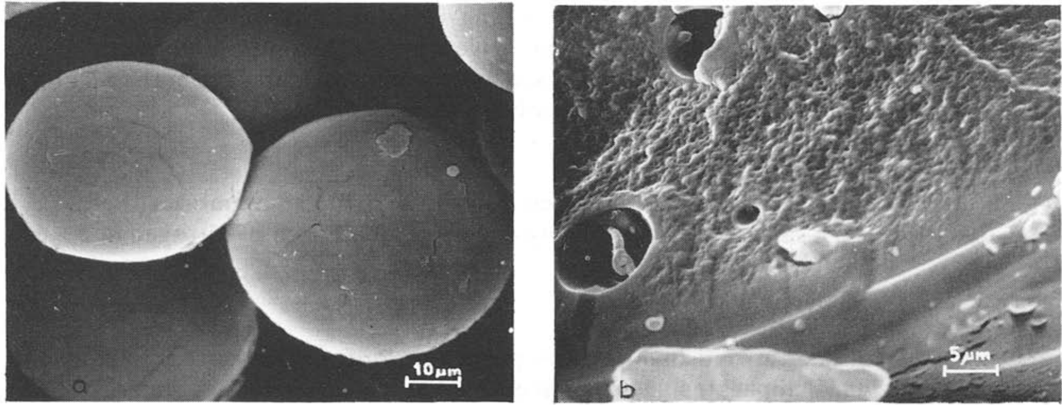

Fig. 2. Scanning electron microphotograph of Bio-Gel P-100 beads. Details as in Fig. 1.

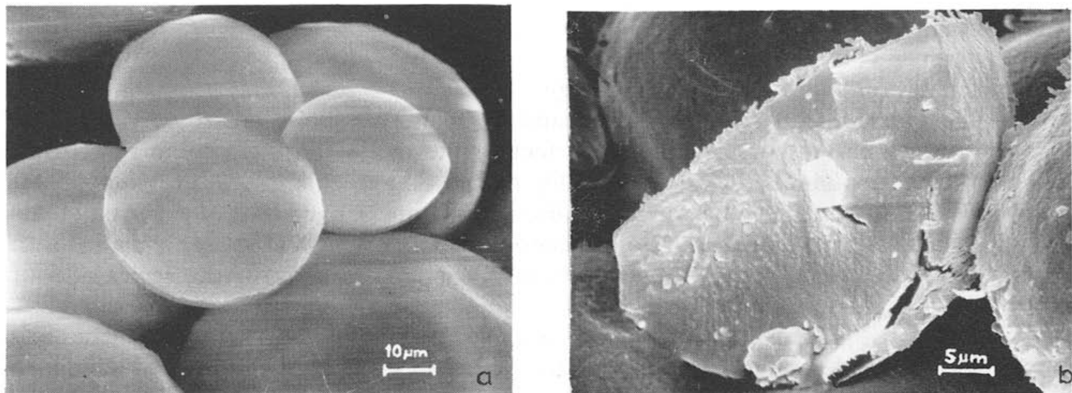

Fig. 3. External microscopic structure of Ultrogel AcA-44. Details as in Fig. 1.

which creates a folded or crinkled aspect, forms during drying of the beads by a contraction of the surface layer which is apparently very soft[16]. The internal structure of Bio-Gel P consists of two zones, a very dense superficial zone and a central portion which contains a microgranular structure. The interior of Ultrogels AcA seems to be fairly homogeneous with a microgranular aspect, but without an apparent division into two zones, as with Bio-Gels.

A parallel study on dry agarose[17] showed that Ultrogels had a greater resemblance to agarose gels than to polyacrylamide gels.

The magnifications employed enabled us only to obtain a general view of the more or less porous structure of the gels. We thus undertook measurements of the surface area and porosity.

Measurements of specific area

The results obtained with the BET nitrogen thermal-desorption method (Table I) show that there are considerable differences between the Ultrogels AcA and the other gels studied. The three types of Sephadex G, as well as the three types of

TABLE I

DETERMINATION OF SPECIFIC AREA BY THE BET THERMAL-DESORPTION METHOD

Gel	*Area (m^2/g)*	*Degassing conditions*	*Weight loss during degassing (%)*
Sephadex G-75 (Lot 1866)	< 0.5	35 h at 50°	9.45
Sephadex G-150 (Lot 9890)	0.17	18 h at 55°	9
Sephadex G-200 (Lot 462)	< 0.5	35 h at 50°	10.84
Bio-Gel P-60 (Lot 11776)	< 0.5	35 h at 50°	10.34
Bio-Gel P-100 (Lot 74634)	< 0.5	35 h at 50°	8.54
Biol-Gel P-300 (Lot 43273)	< 0.5	35 h at 50°	12.01
AcA-22 (Lot 9743)	12.5	5 h at 60°	19.25
AcA-34 (Lot 3013)	1.5	5 h at 55°	16.05
AcA-44 (Lot 2526)	158	18 h at 55°	13.47
AcA-54 (Lot 5006)	236	5 h at 55	7.05

Bio-Gel P, have a very low specific area, < 0.5 m^2/g. Ultrogels AcA, however, present a surprisingly large specific area which, at a constant agarose concentration (AcA-34, AcA-44 and AcA-54), increases with increasing acrylamide concentration to 236 m^2/g.

With these results in mind, the question arose as to the reproducibility of measurements on the same batch and the reproducibility of the specific area between different batches of the same type of Ultrogel AcA. The area measurements were reproducible, as was the weight loss during degassing (Tables II and III). For inter-batch measurements of specific area, it was shown for AcA-54 that, even though there were considerable variations, the specific area is relatively large and is consistently > 100 m^2/g.

TABLE II

REPRODUCIBILITY OF SPECIFIC AREA MEASUREMENTS FOR ULTROGEL AcA-54 (LOT 9776)

Area (m^2/g)	*Degassing conditions*	*Weight loss during degassing (%)*
98	18 h at 45–52°	11.2
99.5	18 h at 45–52°	12.1
103.7	18 h at 56–62°	11.95

TABLE III

DETERMINATION OF THE SPECIFIC AREA OF THREE DIFFERENT LOTS OF ULTROGEL AcA-54

Lot	*BET area (m^2/g)*	*Degassing conditions*	*Weight loss during degassing (%)*
5006	236	5 h at 55°	7.05
5082	159	5 h at 60°	15.9
9776	103.7	18 h at 56–62°	11.95

We also observed relatively high specific areas for the other Ultrogels AcA, with the exception of Ultrogel AcA-22, but with a less satisfactory reproducibility. This variability may be explained solely on the basis of a poor reproducibility of the dehydration method; it is difficult to attribute this dispersion to polymerization differences, since the gel-filtration characteristics of various batches are perfectly comparable.

Porosity studies

The mercury-penetration method enabled us to determine the porous volume, the porous surface and the pore distribution of the various types of gels. These results (Table IV) show that the porosity of Sephadex and Bio-Gels P increase with increasing exclusion limits. The pore distribution, however, is very different for these two types of gels. The curve for Sephadex G has two maxima (Fig. 4), the first located at *ca.* 100 Å and the second at a pore diameter of *ca.* 2 μm. This second peak corresponds to the craters observed during electron microscopy. This porosity is ineffective for the molecular sieving of proteins. The pore distribution of Bio-Gel P is regular, with a maximum at < 100 Å.

TABLE IV

DETERMINATION OF THE POROUS VOLUME OF THE THREE TYPES OF GEL WITH A MERCURY POROSIMETER

Gel	*Porous volume (cm^3/g)*	*Fractionation range (daltons)*
Sephadex G-75 (Lot 1866)	0.01	3,000– 70,000
Sephadex G-150 (Lot 9890)	0.02	5,000– 400,000
Sephadex G-200 (Lot 462)	0.025	5,000– 800,000
Bio-Gel P-60 (Lot 11776)	0.009	3,000– 60,000
Bio-Gel P-100 (Lot 74634)	0.03	5,000– 100,000
Bio-Gel P-300 (Lot 43273)	0.05	60,000– 400,000
AcA-54 (Lot 9776)	2.22	5,000– 70,000
AcA-44 (Lot 2526)	0.64	10,000– 130,000
AcA-34 (Lot 3013)	0.02	20,000– 350,000
AcA-22 (Lot 9743)	0.05	100,000–1,200,000

Ultrogels AcA are much more porous than the other gels studied. AcA-22 and AcA-34 exhibit porosities in the range 50–200 Å, while AcA-44 and AcA-54 have pore diameters which are much greater than 100 Å (up to 2500 Å for AcA-54). This type of porosity is attributed to the presence of agarose at the moment of polymerization of the acrylamide, creating a microgranular network whose structure and size depend on the concentration of acrylic monomer.

DISCUSSION AND CONCLUSIONS

The physical studies presently performed on gels used for protein chromatography show that the dry state of these gels may, depending on the drying method used, lead to the appearance of artifacts as described by Belavtseva *et al.*[18]. There exists a controversy in the literature concerning polyacrylamide gels[19–21]. Certain

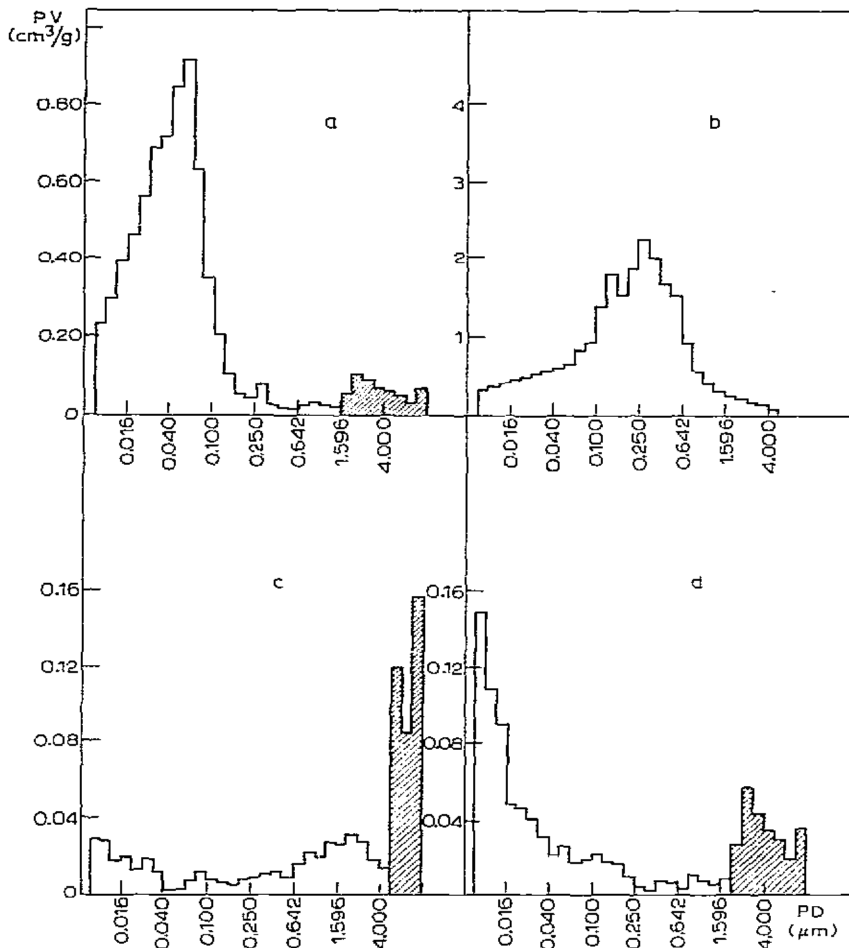

Fig. 4. Pore distribution and porous volume of Ultrogel AcA-44 (a), AcA-54 (b), Sephadex G-150 (c) and Bio-Gel P-300 (d). The hatched areas represent the intergranular volumes. PV = Porous volume (cm^3/g); PD = pore diameter (μm).

workers believe that the cellular structure observed in these gels is due simply to the ice crystals which form during lyophilization. We have performed several tests on lyophilized Ultrogel AcA beads and have indeed observed structural deformations.

Dehydration with organic solvents leads to a retention of the spherical structure of the beads and it was thus possible for us to compare objectively Ultrogels AcA, which are normally sold in a hydrated form, with Sephadex G and Bio-Gels P, sold as dry beads. The comparative electron microscopic observations of these gels showed considerable differences which could sometimes explain certain characteristics of these products when used in the hydrated state. Thus, the alveolar, crinkled aspect of the external portion of Sephadex G beads explains the low mechanical surface resistance and consequently the very low flow-rates and progressive decrease of flow-rates under a hydrostatic pressure, *e.g.*, greater than 10–15 cm of water for Sephadex G-200[22]. It also appears that the crinkled aspect which forms during drying is decreas-

ingly pronounced with increasing degree of gel crosslinking[16]. Thus, the surface of a Sephadex G-10 bead is completely smooth.

In spite of the interesting data furnished by electron microscopy, there is relatively little information concerning the actual porosity available for gel filtration of proteins. Such information, however, shows the degree to which polymers with similar applications may differ from each other. Thus measurements were made of the specific area and porous volume in order to understand more fully the porosity of these gels.

The specific area measurements performed on Sephadex G, Bio-Gel P and Ultrogel AcA demonstrate the existence of very large differences between mixed and homogeneous gels. The surface area of the latter is strongly reduced, while that of mixed gels may reach 100 m^2/g. Although the specific area of Ultrogels AcA may occasionally be extremely elevated, we observed a large variability among gel types. A study of the reproducibility of the drying effect (Tables II and III) leads to values of the specific area which are occasionally quite different, but consistently high (98–236 m^2/g). These differences are primarily a function of the reproducibility of the solvent drying method. Although our measurements do not always represent the absolute specific areas of the gels, they nevertheless furnish relative indications.

When discussing the porous structure of these gels it is important to remember that this property is characterized primarily by the apparent density of the support, the porous volume, the mean pore diameter and the pore size distribution. We may distinguish three types of porosities for gels operating primarily in the swollen state in any solvent[23]:

The gel porosity, which exists only when the three-dimensional macromolecular network is completely swollen in water or the organic solvent.

A porosity which is provided by the pores located between the microgranules and whose aggregation forms the bead. The diameter of these pores is > 250 Å. In the presence of a solvent practically ineffective for gel swelling, only these pores are filled.

The microporosity, which corresponds to the pores present in the microgranules or in the basic particles which form the gel. The diameters of these pores are generally *ca.* 10 and 100 Å. These pores are responsible for the specific internal surface of the gel and the phenomenon of gel filtration.

Measurements of porous volumes by the mercury-penetration method led to complementary data being obtained. The Ultrogels AcA, except AcA-22, are much more porous than the other supports, at least to the extent that this porosity is not present in the same domain. The pore diameters of AcA-22 and AcA-34 are between 50 and 250 Å and those of AcA-44 and AcA-54 are distributed in a zone between 500 and 2000 Å (Fig. 4). This macroporosity is undoubtedly due to the action of agarose on acrylamide polymerization. This phenomenon does not occur with AcA-22 and AcA-34 because the monomer concentrations are undoubtedly too low. The porosity of Sephadex G and Bio-Gels P increases with increasing exclusion limit, with a very different pore distribution. In the case of Sephadex G, the curve exhibits two maxima, at 100 Å and at 1–2 μm (Fig. 4). The second porosity peak corresponds to the craters observed within the gel mass and which do not participate in protein sieving. The

pore size distribution of Bio-Gels P is more regular, with a maximum at < 100 Å. This regularity is undoubtedly related to the uniformity of acrylamide polymerization.

We may compare three different gels which have in common the same domain of globular protein fractionation (Sephadex G-75, Bio-Gel P-60 and Ultrogel AcA-54). The homogeneous gels have an extremely low porosity, *ca.* 0.01 cm^3/g. This is a very significant finding which indicates that dextran and polyacrylamide gels undergo a certain contraction upon drying as a result of their low rigidity. Ultrogels, however, have a much more rigid structure and retain a large proportion of their initial porosity, thus leading to more reliable results.

In conclusion, this study has enabled us to show that considerable structural differences exist among gels intended for the same use. Although these differences only partially explain the variations in behaviour of these supports in protein filtration, we could nevertheless show that the preparation of mixed gels such as Ultrogels leads to a greater porosity than the other types of gels currently available. The prime advantage of this higher porosity is that the mixed gels have a greater mechanical resistance. This particular characteristic of Ultrogels AcA makes it possible to separate protein mixtures more rapidly with no significant decrease of separation efficiency[24].

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