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Application of colloidal gold for characterization of supports used in size-exclusion chromatography

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ABSTRACT

Calibration of matrices for size-exclusion (gel permeation) chromatography (SEC) has to be done for the characterization of supports with inert pores and to allow the determination of the molecular masses of macromolecules. It has been shown by several groups that for steric reasons SEC is very sensitive to conformational details of macromolecules (*e.g.*, rods or random coils). Therefore, no set of particles, organic macromolecules or biomacromolecules such as proteins exists which is universally applicable for calibration and characterization of chromatographic supports. It is shown that colloidal gold, which behaves in SEC as macromolecules, offers the advantage of hydrophilic particles with ideally spherical form and easily measured diameters. Therefore, collodial gold can be used for monitoring of the SEC process at the electron microscopic level, for the characterization of supports (determination of pore size) and for the determination of exclusion limits of matrices.

INTRODUCTION

Size-exclusion chromatography (SEC) or gel permeation chromatography is a widely used method in analytical and preparative macromolecular chemistry and biochemistry. In a conventional model the assumption is made that SEC is based on partition of macromolecules (solutes) dissolved in the mobile phase in pores of a macroporous support (stationary phase). In a simplified case, this partition depends only on the sizes of the macromolecule and the pores, respectively.

For the characterization of macromolecules or supports, all the solute particles in the mobile phase should have the same shape and should not interact with the matrix, *e.g.*, by ionic or hydrophobic forces. Further, the pores of the matrix should be tubes or caves of uniform cross-section. However, in reality, these criteria are not fulfilled completely. Especially biomacromolecules differ from spherical geometry, even under denaturing conditions (application of buffers with high concentrations of urea or guanidinium hydrochloride), and can change their structure during interaction with the matrix as a consequence of the flexibility of the chain of their elements. Denaturation of proteins by sodium dodedyl sulphate (SDS) mostly converts the more or less globular proteins into rod-like particles, which have much higher Stokes radii than the non-denatured forms and therefore show lower abolute distribution coefficients (K_D) than the native species [1].

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Several attempts have been made to correlate size parameters such as Stokes radii with chromatographic behaviour in SEC [1–3]. In particular, no universal correlation exists between molecular mass (M_r) and Stokes radius (R_s) or between K_D and R_s. To fit the equation $R_s = aM_r^b$, different coefficient a and b were found for dextrans, polyethylene glycols, polyethylene oxides, spherical proteins [4] and inorganic colloids (aluminosilica sols) [5]. Whereas calibration of SEC columns for M_r determination of synthetic polymers with uniform monomers fulfils the theoretical considerations [6], especially at low polymer concentrations, in the area of biomacromolecules the determination of M_r from SEC data is possible only when the unknown and the calibration standards have the same shape under the distinct experimental conditions.

Values for exclusion limits and pore sizes depend strongly on the (bio)macromolecules used for the determination. Theoretical considerations discriminate between hard-sphere solutes, rods and random-flight chains, which interact differently with the pores of a support [7]. Therefore, for the characterization of SEC gels in aqueous mobile phases, standards which are similar and unchangeable in size and shape and which can be simply monitored would be useful.

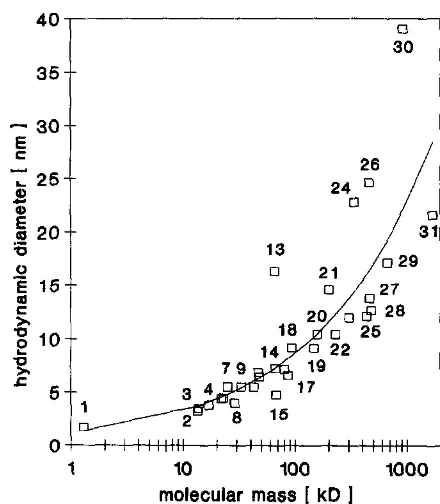

Fig. 1. Plot of hydrodynamic diameter *versus* molecular mass for several macromolecules used in SEC calibration. Macromolecules: 1 = cobalamin; 2 = cytochrome *c*; 3 = RNase II-A; 4 = myoglobin; 5 = trypsininhibitor; 6 = chymotrypsinogen A; 7 = ovomucoid; 8 = carbonic anhydrase; 9 = β-lactoglobulin; 10 = ovalbumin; 11 = Triton X-100 (detergent, micelle); 12 = $C_{12}E_8$ (detergent, micelle); 13 = tropomyosin; 14 = serum albumin; 15 = haemoglobin; 16 = transferrin; 17 = alkaline phosphatase; 18 = tyrosyl-tRNA synthetase; 19 = aldolase; 20 = immunoglobulin G; 21 = α-actinin; 22 = catalase; 23 = aspartate transcarbamylase; 24 = fibrinogen; 25 = ferritin; 26 = spectrin (dimer); 27 = β-galactosidase; 28 = urease; 29 = thyroglobulin; 30 = spectrin (tetramer); 31 = haemocyanin. Data from refs. 1, 2, 8 and 9. kD = kilodalton.

Fig. 1. demonstrates the deviations from a narrow correlation (non-linear regression curve) of molecular mass and hydrodynamic diameter for (bio)macromolecules, frequently used in SEC calibration. These deviations suggest that especially for proteins the M_r determined by SEC should be interpreted very carfully and compared with values obtained by independent methods such as electrophoresis (Ferguson plot analysis), light scattering, viscosimetry, etc.

Application of colloidal gold can overcome some of the disadvantages of organic macromolecules, because the gold particles can be produced as ideal hard-sphere particles, their diameters are easily measurable by electron microscopy, they can be used in aqeuous suspensions and they cannot alter their shape when interacting with the matrix. However, for the determination of molecular masses of biopolymers other standards and distinct correlations are needed.

EXPERIMENTAL

Materials

The supports used were Sepharose 4B (Pharmacia LKB, Uppsala, Sweden) and beaded cellulose Divicell, 80–200 μm (Leipziger Arzneimittelwerk, Leipzig, Germany). A 1% aqueous solution of $HAuCl_4$ was obtained from Feinchemie (Sebnitz, Germany), polyethylene glycol (PEG) 20000, trisodium citrate, tannic acid, bovine serum albumin (BSA) and dinitrophenylalamine (DNP-Ala) were obtained from Serva (Heidelberg, Germany) and Blue Dextran 2000 from Pharmacia (Freiburg, Germany). Chromatography was performed at room temperature in 40 × 1 cm I.D. glass columns using a P1 peristaltic pump and a Uvichord SII UV monitor (Pharmacia). Colloidal gold of different size was prepared according to ref. 10 and was used without further purification.

Methods

Chromatography was done in doubly distilled water containing 0.05% (w/v) PEG 20000 and 0.02% (w/v) of NaN_3, and was monitored at 280 nm. Linear flow-rates were 23.8 cm/h for Divicell and 3.0 cm/h for Sepharose 4B. The sample volume was 1.0 ml, containing 1 absorbance unit of each probe.

For electron microscopy, the beaded cellulose was loaded with the appropriate gold colloid, then it was lyophilized and the spheres were directly embedded in Epon 812. Sections were cut with glass knives on a Ultratome III (LKB). For size determination of colloidal gold, it was spread on 3-mm grids without fixation. Electron microscopic examinations were made on a JEM 100 CX electron microscope (JEOL, Tokyo, Japan).

RESULTS AND DISCUSSION

Fig. 2 shows the electron micrographs of two different colloidal gold preparations. Their photometric properties are given in Table I. Especially the larger material (particle diameter 14 ± 1 nm, Fig. 2A) appears in the electron micrograph as regular spheres. Their homogeneous size distribution is reflected in SEC with both Divicell and Sepharose (Fig. 3A and B, respectively). As shown in Fig. 4, the distribution coefficients (K_{av}) values of gold particles vary linearly with the particle diameter when spheres with diameters from 4 to 14 nm were used. The hydrodynamic diameter and the distribution coefficient of BSA as an example of a globular protein fit this plot of particle diameter *vs.* K_{av} but, as discussed earlier, this cannot be generalized.

The inhomogeneity in size of the gold preparation of 4-nm mean diameter, as shown in the electron micrographs (Figs. 2B and 5B), is reflected in SEC. On both Divicell (Fig. 3A) and Sepharose (Fig. 3B) the relatively large portion of bigger grains appears as an early peak in the chromatograms.

In Fig. 5, the principle of gel filtration becomes visible: exclusion or low penetation of the large gold particles and deep penetration of the smaller particles into the chromatographic matrix (beaded cellulose). As a real SEC matrix contains pores with a non-uniform distribution of diameters and the diffusion rate is inversely proportional to the molecular radius, differently sized particles can penetrate the matrix more or less and prolong the time needed to leave the column.

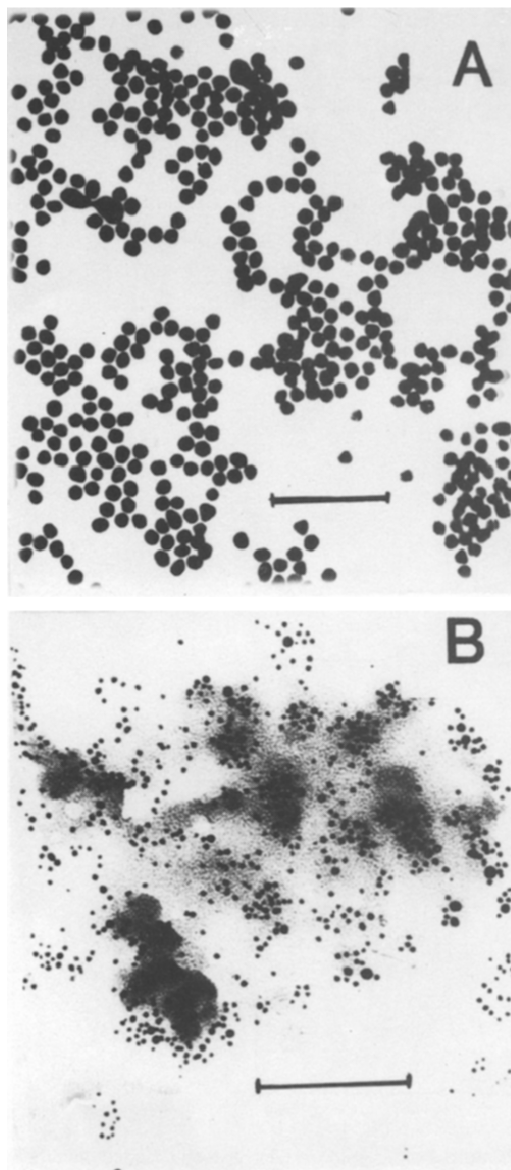

Fig. 2. Electron micrographs of colloidal gold preparations (original magnification × 85 000). (A) Particle diameter 14 ± 1 nm, larger particles up to 28 nm < 5%; (B) particle diameter 4 ± 1.5 nm, larger particles up to 10 nm ≈ 20%. Bars represent 100 nm.

TABLE I

ABSORPTION COEFFICIENTS OF COLLOIDAL GOLD PREPARATIONS

The values obtained are combinations of light absorption and scattering, but registered as absorptions in the two-beam photometer used. Values calculated for 0.1 mg/ml of Au, 1 cm path length.

Sample[a]	$A_{280\ nm}$	$A_{\lambda max}$	λ_{max} (nm)
Au_4	3.505	0.975	515
Au_6	1.841	0.881	526
Au_{10}	1.291	1.133	519
Au_{14}	0.801	0.852	520

[a] Subscripts are mean particle diameters (nm).

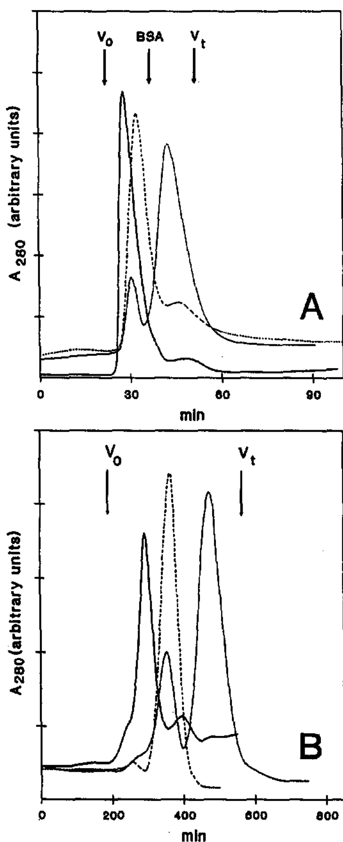

Fig. 3. SEC of colloidal gold on (A) Divicell (beaded cellulose) and (B) Sepharose 4B (beaded agarose). Elution profiles for the respective gold sols: solid lines, Au_{14}; dashed lines, Au_{10}; dotted lines, Au_4 (subscripts represent the mean particle diameters in nm). V_0, elution volume for Blue Dextran 2000 (void volume); BSA, elution volume for bovine serum albumin; V_t, elution volume for DNP-Ala. (total volume).

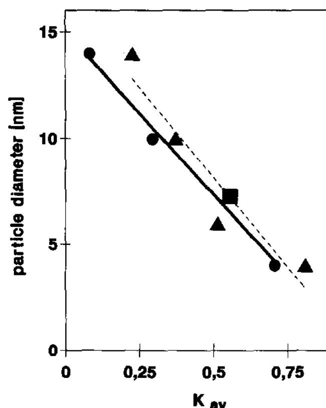

Fig. 4. Plot of K_{av} *versus* particle diameter for colloidal gold, estimated on Divicell (●, solid line) and Sepharose 4B (▲, dotted line). ■ = Respective hydrodynamic and K_{av} values for BSA, run on the same column.

Although, in the uncontrasted electron micrograph, the matrix is only poorly visible, in Fig. 5A it is clearly demonstrated that the large gold particles (Au_{14}) are located at or near the surface. In contrast, the small gold particles (Au_4) are also found in deeper layers of the matrix. Fig. 5C illustrates schematically the principle of the electron micrographs. Hence these figures visualize the principle of gel filtration.

Another interesting effect, first observed in affinity chromatography, can also be seen in Fig. 5B, *viz.*, the density of the gold particles decreases in the direction from the surface to the centre of the cellulose bead. This formation of a "shell" has been found, for instance, when antibodies [11] and enzymes [12] were covalently immobilized on chromatographic supports. Probably this shell may be influenced by the concentration of the solute and/or the flow-rate of the eluent, and its existence could indicate that in a real experiment equilibrium of the solute distribution between the mobile and stationary phases is not reached.

Laboratory-made or commercially available gold particles with diameters ranging from 5 to 30 nm offer the opportunity of determining exact pore size distributions of hydrophilic gels and therefore of true exclusion limits (particle sizes excluded from the matrix). Previously, this was mostly done by using synthetic polymers such as polystyrenes or dextrans [13] or by calibration with globular pro-

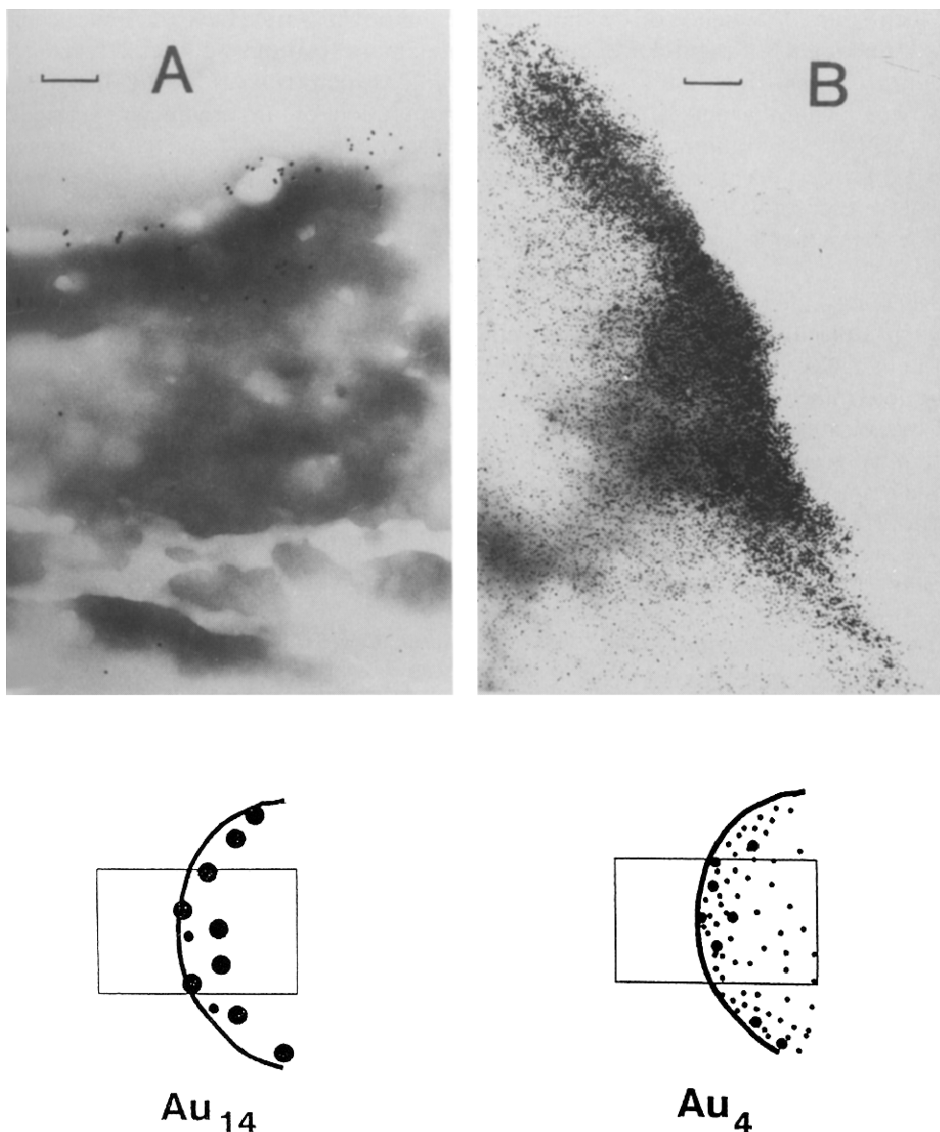

Fig. 5. Transmission electron micrograph (original magnification × 58 000) of partial exclusion of larger particles (mean diameter 14 nm) from Divicell beads (A) and complete penetration of colloidal gold (mean diameter 4 nm) into the matrix (B). (C) Schematic representation of (A) and (B), respectively. The rectangles illustrate the cut of the surface area of cellulose beads as shown in (A) and (B). Bars represent 200 nm.

teins. Calibration with organic macromolecules suffers from the flexibiliy of the macromolecular structures [7], their differing deviations from spherical shape and from several possible types of interactions with the chromatographic matrix. Colloidal metals allow some of these unwanted side-effects to be overcome and the chromatographic behaviour of the colloid and of the matrix can be monitored by (electron) microscopy.

Limitations on the use of colloidal metals are set

by their salt sensitivity, which is a consequence of electrostatic repulsive forces making a sol possible and which requires working at very low ionic strengths. However, as matrices such as beaded cellulose (Divicell) or agarose (Sepharose) contain only very few negatively charged groups, exclusion of (negatively charged) gold sol from pores with diameters in the range of particle diameters is not very probable.

Stabilization of gold colloids by adsorption of proteins or other polar macromolecules is accompanied by an increase in the hydrodynamic diameters [14], which are then not determined as simply as for the pure particles. Further, because this coating is the result of an equilibrium between coated particles and free macromolecules, this macromolecular shell has to be maintained during the whole chromatographic process. Moreover, using a mobile phase as described under Experimental, the simple procedure, the easy monitoring and the general applicability to diverse hydrophilic gels make the use of colloidal metals, especially colloidal gold, recommended for the characterization fo SEC columns, but the problem of calibration for molecular mass determinations of proteins has not been solved, because it is established in the diversity of biopolymers.

CONCLUSIONS

The application of collodial gold for the characterization of SEC supports has advantages and disadvantages compared with other materials used for calibration in SEC. The main disadvantages are chromatographic applicability only at low ionic strengths and the impossibility of determining molecular masses in a simple way from chromatographic data as discussed above. As with other calibration standards, this is possible only if the standard and the unknown sample have the same geometry–molecular mass relationship. The advantages of colloidal gold, which behaves in SEC like a homogenous population of an organic macromolecule, are the easily determined diameter of the gold particles, the homogeneity of the diameter in a given preparation or the possibility of determining the size distribution by electron microscopy, the chemical stability in aqueous solutions and the simple monitoring of the chromatographic process by measuring the optical density in the UV or visible wavelength range.

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