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STERIC PROTECTION OF A MODIFIED GLASS SURFACE WITH ADSORBED POLYACRYLAMIDE: INTERACTIONS WITH BLOOD PROTEINS

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SUMMARY

Glass beads chemically modified with aluminol (AlOH) groups were coated with a monolayer of high-molecular-mass polyacrylamide at different temperatures and exposed to ^{125}I-labelled albumin and fibrinogen solutions for 1 h. No adsorption of albumin was observed, whereas fibrinogen adsorption increased as a function of temperature. These observations were interpreted in terms of the density and stability of the adsorbed polymer layer. At 25 °C, where fibrinogen adsorption was already important, it was shown, using ^{3}H-labelled polyacrylamide, that fibrinogen partly displaced the polymer from its adsorption sites.

INTRODUCTION

To separate synthetic and biological macromolecules according to their size and/or chemical composition, chromatographic techniques have been used for several decades. Porous silica is the most popular packing material, and its use with polar aqueous mobile phases has been developed by effecting interfacial chemical modifications of this stationary phase. Gilpin [1] reviewed the properties of the so-called bonded phases obtained via interfacial chemical bonding of low-molecular-mass organic solutes. An alternative method to the modification of interfacial properties is to deposit or adsorb polymers at the solid–liquid interface and, since the early work of Boardman [2,3], many results have been published describing numerous coating materials and methods [4–19].

Steric exclusion chromatography allows the separation of the macromolecules

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according to their size (or hydrodynamic volume), and with water as solvent it is extensively used in so-called gel permeation media, where only limited overpressures can be applied. High-performance liquid chromatography with solid (and therefore non-compressible) packings is a technique that is still in its research and developmental phase, especially with regard to the separation of hydrosoluble macromolecules of intermediate or high molecular mass [20].

A steric exclusion mechanism requires non-adsorbing solid surfaces with respect to the macromolecules dissolved in the aqueous phase. However, it is well known that non-specific adsorption is commonly encountered, especially with proteins [20,21]. Therefore, to achieve the steric protection of adsorbing surfaces, physical adsorption of hydrosoluble or at least partly hydrophilic polymers or copolymers seems to be a good approach. The method provides dense interfacial macromolecular coatings, playing the role of an elastic barrier to prevent further adsorption. However, short- and long-term stability problems remain to be explored. It has been shown that macromolecular adsorption is generally irreversible with respect to dilution of the adsorbing solution, but exchange between adsorbed and dissolved molecules has been observed with both synthetic and biological polymers [22–24]. Consequently, to prepare surfaces sterically protected by an adsorbed polymer layer, the stability of the layer has to be checked and eventually improved.

The aim of this paper is to present experimental results obtained with chemically modified glass beads, coated with an adsorbed layer of high-molecular-mass polyacrylamide. Polyacrylamide gels are currently used in electrophoretic separations, as the polymer displays little interaction with proteins. Adsorption and desorption of this polymer have recently been studied in our laboratory [24–26] and we have now investigated protein adsorption and the stability of the adsorbed polyacrylamide layer when it is exposed to protein solutions.

EXPERIMENTAL

Polymer

Polyacrylamide was prepared by radical copolymerization of acrylamide mixed with small amounts (0.1%, mol/mol) of acrolein. After polymerization, the polymer was purified, fractionated and radiolabelled in basic aqueous medium (pH 9.5) with tritiated potassium borohydride. The sample we used displayed the following characteristics: molecular mass, MW, $1.2 \cdot 10^6$; polydispersity index (GPC), 1.25; and specific radioactivity, $1.12 \cdot 10^8$ counts $min^{-1}\ g^{-1}$. Further details have been published elsewhere [24].

Adsorbent

Non-porous glass beads with a mean diameter of 47 μm and a specific surface area of 0.058 $m^2\ g^{-1}$, measured using a coulter counter model TA2 [27], were first hydroxylated by soaking for several hours in boiling 1 *M* hydrochloric acid. The material was then thoroughly washed and dried before treatment with aluminium chloride dissolved in chloroform. Silanol groups (SiOH) were thus partially replaced by aluminium chloride groups (AlCl). Subsequent hydrolysis led to an

interface partially covered with aluminol (AlOH) groups, the ratio [AlOH]/[SiOH] as determined by potentiometric titration [28] being close to 0.12.

Experimental system

The modified glass beads were packed into a "mini-column" made of polytetrafluoroethylene tubing (8 cm × 3 mm I.D.) fitted at both ends with a filter (10 μm pore diameter) and equipped with Gilson-type connectors. This column was placed in a Plexiglas envelope thermostated to within ±0.2°C by a Haake F2-6 thermostat.

Solutions (polymer, buffer, protein) were injected with a speed-adjustable syringe pump into the column placed over a γ-scintillation crystal (NaI; Quartz et Silice). The photomultiplier was coupled to a multi-channel analyser (Cosynus 60; Enertec Schlumberger), which permitted continuous counting when a ^{125}I-labelled protein solution was injected. With the ^{3}H-labelled polymer, fractions were collected at the outlet of the column and counted using a liquid scintillator (Intertechnique SL 2000; Instagel Packard).

Protein and buffer solutions

Human fibrinogen (grade L) was purchased from Kabi, prepared in 1-ml aliquots at a concentration of 1% (w/w) and stored at −30°C. Immediately before an experiment, it was rapidly thawed at 35°C and diluted with the buffer. Purified human albumin was obtained from the Centre Régional de Transfusion Sanguine (Strasbourg, France) and handled under the same conditions. Both proteins were labelled with ^{125}I by the iodine monochloride method [29,30] and dissolved in a 0.05 *M* Tris–0.15 *M* sodium chloride buffer solution at pH 7.35.

Procedure

In a typical protein adsorption experiment, an aqueous solution of polyacrylamide at pH 4.5 and concentration $5 \cdot 10^{-4}$% (w/w) was circulated for 2 h through the column filled with modified glass beads, at a given temperature. After rinsing with distilled water or Tris buffer solution at pH 7.35, a protein solution at the same pH and temperature and at a concentration of $2 \cdot 10^{-2}$% (w/w) was injected at a flow-rate of 10 ml h^{-1} for 1 h, and subsequently displaced by the buffer solution. To determine the amount of protein remaining adsorbed, the adsorbent was extracted from the column, dried and weighed. Its radioactivity was measured with a Kontron γ-counter and compared with the activity of a known amount of protein solution. The interfacial protein concentration (expressed in mg cm^{-2}) could thus be calculated and compared with the value for bare glass beads ($8.5 \cdot 10^{-4}$ mg cm^{-2} of adsorbed fibrinogen).

RESULTS AND DISCUSSION

Polymer adsorption

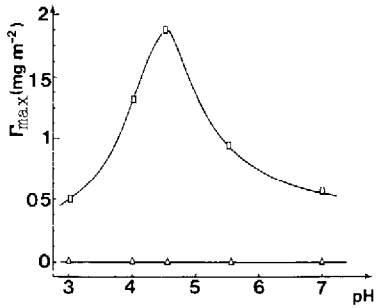
The main parameters governing polyacrylamide adsorption when an aqueous solution comes into contact with an adsorbent surface are solution concentration,

solution pH and temperature. It has been shown [24] that adsorption isotherms are of the high-affinity type. Hence quasi-plateau interfacial concentrations are obtained with equilibrium solution concentrations close to 10^{-4}%, whereas for solutions of proteins such as albumin or fibrinogen these bulk concentrations are two orders of magnitude higher. Therefore, choosing for the polyacrylamide solution flowing through the column a concentration of $5 \cdot 10^{-4}$% (w/w) ensures that the system remains in the domain of plateau adsorption.

Pefferkorn et al. [24,25] showed that plateau polyacrylamide adsorption on the beads under study is strongly pH dependent. Maximum adsorption is reached at pH 4.5 (Fig. 1) where the amphoteric so-called aluminol groups (by analogy with silanol groups) are non-ionized and are therefore able to form multiple hydrogen bonds with the amide groups of the polymer. In subsequent experiments, the preparation of the polyacrylamide-coated interfaces was always performed at a solution pH of 4.5, which is the isoelectric point of the material, obtained by a microelectrophoretic technique [31] (Mark 2; Rank Brothers).

Temperature is the other parameter governing plateau adsorption. Fig. 2 shows that polyacrylamide adsorption increases markedly as the temperature decreases from 55 to 15 °C. This observation correlates well with the thermal behaviour of many polymer–solvent–adsorbent systems, for which the adsorbance increases as the solvent quality decreases towards the so-called θ solvent conditions [32,33]. Polyacrylamide conformational studies in solution [34] show that the quality of the aqueous solvent decreases with decreasing temperature to the extrapolated Flory θ temperature conditions. Moreover, the jump in the adsorption, observed at ca. 40 °C, may be tentatively attributed to the formation of a soft gel-like interfacial structure, stabilized by intra- or intermolecular hydrogen bonds. Such specific attractive interactions generally induce, within one- or three-dimensional systems, helix–coil transitions (proteins, polypeptides) or gel–sol transitions (gelatin, i.e., collagen solution) [35].

To characterize better the polyacrylamide-adsorbed layers, an additional ob-



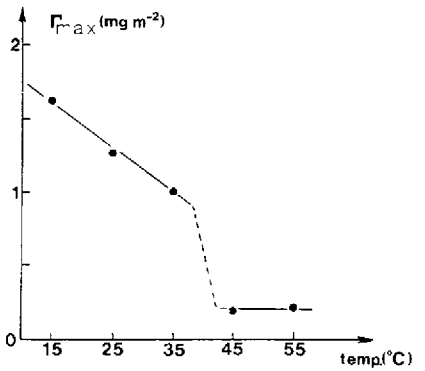


Fig. 1. Variation of the polyacrylamide plateau adsorbance, Γ_{max}, as a function of the pH of the equilibrium solution at 25 °C, on surface-modified glass beads (□) or on unmodified glass beads (no adsorbance) (△).

Fig. 2. Variation of the polyacrylamide plateau adsorbance, Γ_{max}, as a function of the temperature of the equilibrium solution, maintained at pH 4.5.

servation is required. When adsorption measurements are combined with hydrodynamic thickness data, it is possible to calculate the mean monomer concentration within the adsorbed layer and to compare it to the corresponding concentration within the dissolved polymer coil [36]. We found that for the polymer under study, at 25°C and pH 4.5, this ratio is close to 25, showing that the monomolecular layer should provide, with its thickness of 1100 Å [36], good steric protection of the surface.

Protein adsorption on pre-coated surfaces

To analyse the interaction of the coated surface under study with water-soluble proteins, especially blood proteins, we injected, in exploratory work, an albumin solution into a column containing beads coated at 25°C with polyacrylamide, under the conditions already described. Almost no albumin adsorption was detected. This promising result nevertheless required confirmation with other proteins.

Because, as a general rule in non-specific adsorption, macromolecular affinity for an interface is an increasing function of molecular mass [32,33], we decided that fibrinogen (MW = 340 000) was an appropriate protein. Several studies related to the so-called Vroman effect have demonstrated a high surface affinity of this particular plasma protein, especially for glass surfaces [37]. Equilibrium [38] and kinetic [23] experiments comparing the properties of albumin and fibrinogen interacting with heparin-like materials also showed the preferential adsorption of the fibrinogen molecule. Therefore, the study of its interactions with pre-coated beads should provide a severe test of the polyacrylamide–protein competition for the modified glass bead surface.

A typical curve, recorded for injection of protein solution at 8°C into the pretreated minicolumn, is shown in Fig. 3. A rapid increase characteristic of the

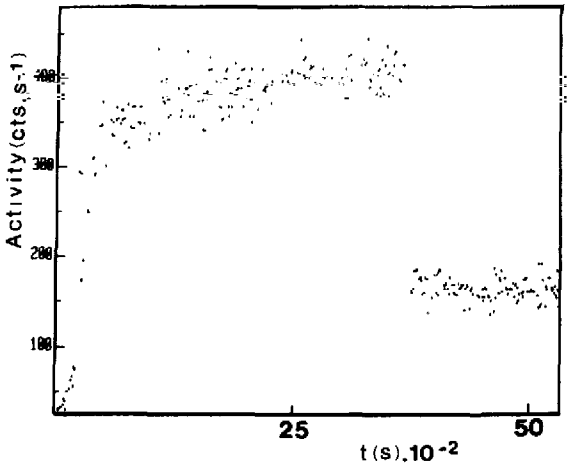


Fig. 3. Variation of the recorded radioactivity within a column containing glass beads pre-coated with polyacrylamide, when a fibrinogen solution is first injected and then replaced by a buffer solution, at 8°C and pH 7.35.

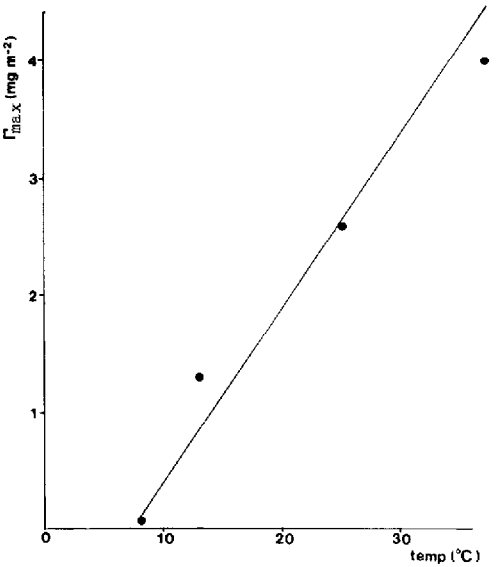


Fig. 4. Fibrinogen adsorption maxima as a function of temperature at pH 7.35 on glass beads precoated with polyacrylamide.

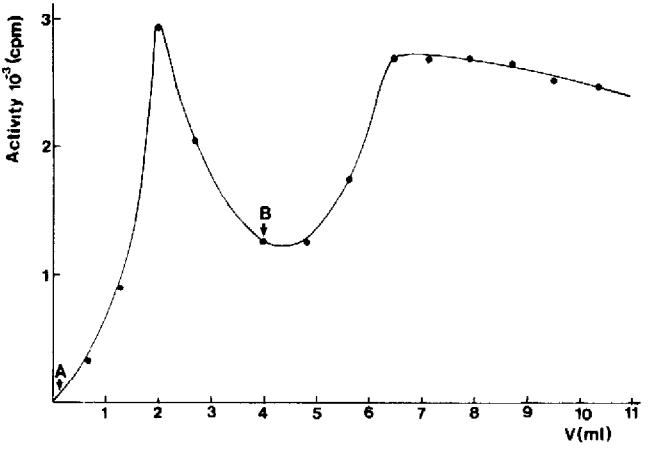


Fig. 5. Desorption of ^{3}H-labelled polyacrylamide preadsorbed on glass beads at 25 °C from an aqueous solution at pH 4.5. After displacement of the polyacrylamide solution by deionized water, water is replaced at (A) by Tris–HCl buffer and at (B) by a solution of unlabelled fibrinogen in the same buffer.

interstitial solution is followed by a limited increase due to adsorption. After 1 h, buffer solution was circulated through the column. A plateau is quickly reached and the remaining radioactivity measures the amount of adsorbed protein.

Fig. 4 illustrates the variation of the amount of adsorbed fibrinogen with operating temperature, which is the same for polymer preadsorption and protein adsorption. It is observed that fibrinogen adsorption decreases markedly with decreasing temperature, a result that is due primarily to the higher density and

stability of the polyacrylamide layer at low temperature. In addition, as the solvent quality decreases and approaches the θ temperature conditions (θ is close to $-5\,^{\circ}C$ for the polyacrylamide–water system), concentration fluctuations within the adsorbed layer become less important and therefore prevent contact between bulky protein molecules and the adsorbing surface. At an operating temperature of $8\,^{\circ}C$, the amount of adsorbed fibrinogen is only 2% of the amount measured at $25\,^{\circ}C$ on untreated glass beads. If we take into account the fact that the residence time of a "concentration pulse" in a column is much less than 1 h, it is seen that, at low temperature, a polyacrylamide layer provides good steric protection of the glass surface with respect to protein adsorption.

Finally, to investigate polymer–protein competition in interfacial affinity, we performed an experiment with ^{3}H-labelled polymer preadsorbed on the beads, at $25\,^{\circ}C$, from an aqueous solution at pH 4.5 and a concentration of $5 \cdot 10^{-4}\%$ (w/w). After contact with the column for 2 h, the solution was displaced by deionized water and no desorption was recorded, as already reported by Pefferkorn et al. [24] and Carroy [26]. However, when water was replaced by the Tris buffer solution, part of the adsorbed polymer (about 15%) was quickly desorbed, as shown in Fig. 5. Such partial desorption induced by a change of the fluid phase is commonly observed in chromatography. Subsequent injection of a non-labelled fibrinogen solution at concentration of $5 \cdot 10^{-2}\%$ (w/w) induced additional and important desorption. This is a surprising result, because on comparing the adsorption isotherms of the two types of macromolecules, the higher affinity of the polyacrylamide molecule for the interface is evident [36]. However, such simple arguments may not hold when dealing with competitive adsorption between flexible macromolecules and bulky protein molecules.

CONCLUSION

Modification of the interfacial properties at a solid–liquid interface may be achieved by chemical grafting at the solid surface. This procedure usually leads to diffuse layers which are not sufficiently dense to sterically protect the surface. In addition, these layers are not easily characterized. Conversely, physical adsorption of well characterized macromolecules is a potentially promising approach, as dense interfacial layers are obtained. However, the long-term stabilization of such monolayers is not yet satisfactory. The few experimental results that have been presented illustrate the problems that have to be faced in the preparation of non-adsorbing solid phases of potential interest for chromatography.

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REFERENCES

1 R.K. Gilpin, J. Chromatogr. Sci., 22 (1984) 371.
2 N.K. Boardman, J. Chromatogr., 2 (1959) 388 and 398.
3 N.K. Boardman, Biochim. Biophys. Acta, 18 (1955) 290.
4 C.G. Horvath, B.A. Preiss and S.R. Lipsky, Anal. Chem., 39 (1967) 1422.
5 J.J. Kirkland, J. Chromatogr. Sci., 8 (1970) 72.
6 C.W. Hiatt, A. Shelokov, E.J. Rosenthal and J.M. Galimore, J. Chromatogr., 56 (1971) 362.
7 G.L. Hawk, J.A. Cameron and L.B. Dufault, Prep. Biochem., 2 (1972) 193.
8 I. Schechter, Anal. Biochem., 58 (1974) 30.
9 T. Darling, J. Albert, P. Russel, D. Albert and T W. Reid, J. Chromatogr., 131 (1977) 383.
10 J.L. Tayot, M. Tardy, P. Gattel, R. Plan and M. Roumiantzeff, in R. Eptom (Editor), Chromatography of Synthetic and Biological Polymers, Vol 2, Ellis Horwood, Chichester, 1978, pp. 95–110.
11 A.J. Alpert and F.E. Regnier, J. Chromatogr., 185 (1979) 375.
12 B.S. Welinder, J. Liq. Chromatogr., 3 (1980) 1399.
13 T. Mizutani, J. Chromatogr., 196 (1980) 485.
14 D.L. Gooding, M.N. Schmuck and K.H. Gooding, J. Chromatogr., 296 (1984) 107.
15 L. Letot, J. Lesec and C. Quivoron, J. Liq. Chromatogr., 4 (1981) 1311.
16 E. Pefferkorn, Q.K. Tran and R. Varoqui, J. Chim. Phys., 78 (1981) 549.
17 A.J. Alpert, J. Chromatogr., 359 (1986) 85.
18 R.M. Chicz, Z. Shi and F.E. Regnier, J. Chromatogr., 359 (1986) 121.
19 H. Figge, A. Deege, J. Köhler and G. Schomburg, J. Chromatogr., 351 (1986) 393.
20 G. Seipke, H. Müllmer and U. Grau, Angew. Chem., Int. Ed. Engl., 25 (1986) 535.
21 J.L. Brash and T.A. Horbett (Editors), Proteins at Interfaces. Physicochemical and Biochemical Studies, ACS Symposium Series, No. 343, American Chemical Society, Washington, DC, 1987.
22 J.L Brash, S. Uniyal, C Pusineri and A. Schmitt, J. Colloid Interface Sci., 95 (1983) 28
23 J.C. Voegel, N. de Baillou and A. Schmitt, Colloids Surf., 16 (1985) 289.
24 E. Pefferkorn, A. Carroy and R. Varoqui, J. Polym Sci., Polym. Phys. Ed., 23 (1985) 1997.
25 E. Pefferkorn, A. Carroy and R. Varoqui, Macromolecules, 18 (1985) 2252.
26 A. Carroy, Thesis, Université Louis Pasteur, Strasbourg, 1986.
27 P.H. Walker and J. Hutka, Division of Soils Technical Paper No. 1. Commonwealth Scientific and Industrial Research Organization, Glen Osmond, Australia, 1971.
28 E. Pefferkorn, L. Nabzar and A. Carroy, J. Colloid Interface Sci., 106 (1985) 94.
29 A.S. MacFarlane, J. Clin. Invest., 42 (1963) 346.
30 K. Krohn, L. Sherman and M. Welch, Biochim. Biophys Acta, 285 (1972) 404.
31 A.C. Jean-Chronberg, personal communication.
32 G.J. Fleer and J. Lyklema, in C.D. Parfitt and C.A. Rochester (Editors), Adsorption from Solution at the Solid/Liquid Interface, Academic Press, New York, 1983, Ch. 4, p. 153.
33 A. Schmitt in A.M. Cazabat and M. Veyssié (Editors), Colloides et Interfaces, Editions de Physique, Paris, 1984, p. 245.
34 M. Duval, Thesis, Université Louis Pasteur, Strasbourg, 1982.
35 C.R. Cantor and P.R. Schimmel (Editors), Biophysical Chemistry. Part III. The Behavior of Biological Macromolecules, Freeman, San Francisco, CA, 1980, pp. 1041–1073.
36 J.D. Aptel, A. Carroy, P. Dejardin, E. Pefferkorn, P. Schaaf, A. Schmitt, R. Varoqui and J.C. Voegel, in J.L. Brash and T.A. Horbett (Editors), Proteins at Interfaces. Physicochemical and Biochemical Studies, ACS Symposium Series, No. 343, American Chemical Society, Washington, DC, 1987, p. 222.
37 P. Wojciechowski, P. Ten Hove and J.L. Brash, J. Colloid Interface Sci., 111 (1986) 455.
38 N. de Baillou, J.C. Voegel and A. Schmitt, Colloids Surf., 16 (1985) 271.