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ORIGINAL CONTRIBUTION

D. Kowalczyk
J.-P. Marsault
S. Slomkowski

Atomic force microscopy of human serum albumin (HSA) on poly(styrene/acrolein) microspheres

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D. Kowalczyk · Dr. S. Slomkowski (✉)
Center of Molecular and Macromolecular Studies
Polish Academy of Sciences
Sienkiewicza 112
90-363 Lodz, Poland

J.-P. Mausault
Université Paris 7
Denis Diderto
Institute de Topologie et de Dynamique des Systèmes
1, rue Guey de la Brosse
75005 Paris, France

Abstract Atomic Force Microscopy (AFM) in the tapping mode was used for the observation of bare poly (styrene/acrolein) P(SA) microspheres and microspheres with attached HSA. Prior to the AFM observations the P(SA) microspheres were immobilized covalently on the surface of quartz slides modified with γ-amino-propyltriethoxysilane. Atomic Force Microscopy pictures were registered for the dry samples. The partial coalescence of the P(SA) microspheres connected to the quartz surface with amino groups has been observed. The AFM pictures of the single P(SA) microspheres revealed that the surface of these particles is smooth and that any irregularities, if present, do not exceed 1 nm. The surface of microspheres with attached HSA has very clearly different morphology with regular pattern of HSA macromolecules. Cracks on the surfaces of some microspheres with HSA revealed that protein macromolecules are attached to these particles in several layers. In the case of some other microspheres the defects in protein attachment allowed the observation of the border between the bare surface of the P(SA) microspheres and the surface covered with protein macromolecules. Comparison of the thickness of the HSA layers on the P(SA) microspheres with the dimensions of HSA macromolecules, determined earlier from the x-ray studies, suggests that the first layer, 3.0 ± 0.2 nm thick, is formed of the HSA macromolecules arranged flatly on the surface whereas protein macromolecules in the subsequent layers, each 8.6 ± 1 nm thick, are adsorbed protruding from the surface.

Key words Atomic force microscopy – human serum albumin – poly (styrene/acrolein) microspheres

Introduction

Interest in protein–polymer interactions results from the importance of these phenomena for application of polymers in medicine and biotechnology, e.g. as implants [1], elements of therapeutical and diagnostic equipment [2–6], supports for the immobilization of enzymes, and the whole cells [7]. Investigations of the protein–polymer interactions are essential also for the fundamental studies of the behavior of complex macromolecules with determined internal structure at the liquid–solid interface.

Studies of the protein–polymer interactions include determination of the relation between the nature of the polymeric surface and the surface concentration of adsorbed and/or covalently immobilized proteins, thickness of the protein layer, orientation of attached protein macromolecules, protein denaturation and changes of the biologic activity of protein macromolecules induced by interactions with polymeric materials [8–11]. Some

authors suggested that under specific experimental conditions proteins could be attached in more than one layer [12, 13]. Until now the majority of information on the orientation of protein macromolecules at interfaces was obtained in an indirect way. Computer simulation was also used for modeling protein adsorption to solid supports [14].

Recent developments in scanning probe microscopy provided important tools for direct observation, with molecular resolution, of protein and nucleic acid macromolecules at interfaces [15]. Protein and nucleic acid macromolecules were observed on a conducting (e.g. gold) substrate by Scanning Tunneling Microscopy (STM) [16–19]. Atomic Force Microscopy (AFM) allowed monitoring protein macromolecules on nonconducting supports [15–20, 21]. However, one has to be aware that registration of a picture by AFM in the contact mode could result in the translocation of protein macromolecules on the surface [22–24].

In the studies mentioned above, Atomic Force Microscopy has been used usually for monitoring proteins, nucleic acids, and other biologically important molecules deposited on flat and smooth surfaces. The importance of polymeric latexes and microspheres for medical diagnostics and drug delivery systems drew our attention to the conditions allowing observation of protein macromolecules on the surface of these particles. However, observations of proteins on microspheres could be difficult due to the combination of the curvature and local geometry of the surface of microspheres with the geometrical characteristics of attached protein macromolecules. Moreover, any adventitious movements of a particle onto which proteins were attached could interrupt observation and thus, it would be desirable to immobilize these particles.

In this paper we describe results of observations by AFM of human serum albumin (HSA) attached onto the poly(styrene/acrolein) microspheres. Human serum albumin was selected for our studies because it is most abundant among the blood plasma proteins [25]. The macromolecule of HSA with a molecular weight of 66 500 is organized in three well delineated domains [26–28]. X-ray studies allowed to find out that in the crystal form the three domains of HSA form the heart-like structure with lengths of the sides equal to 8.3 nm, 7.0 nm, and 8.2 nm and with thickness equal to 3.0 nm [27]. Recent studies indicated that the orientation of the adsorbed macromolecules of HSA depends on the nature of the surface (charge and hydrophobicity) and on pH of the medium in which adsorption occurs [29, 30]. However, little is known on the orientation of the covalently immobilized HSA macromolecules.

Poly(styrene/acrolein) microspheres were chosen for the studies because particles with aldehyde groups on the surface are able to immobilize protein macromolecules without any activation, simply by incubation of microspheres and protein [31–34]. In our recent studies we developed the synthetic methods of obtaining monodisperse poly(styrene/acrolein) microspheres with a controlled fraction of polyacrolein in the surface layer [35]. We found also a method suitable for the covalent immobilization of P(SA) microspheres on the quartz slides in two-dimensional assemblies [36]. For these microspheres we investigated the immobilization of HSA, determining the fractions of adsorbed and covalently immobilized protein macromolecules [33, 34]. We expected that such immobilization would be helpful in the case of the observations of attached HSA by the AFM method.

Experimental part

Poly(styrene/acrolein) microspheres (P(SA)) were synthesized in an emulsion–precipitation polymerization of styrene and acrolein initiated with potassium persulfate and carried out without added emulsifier. The polymerization mixture contained: styrene (10 mL), acrolein (0.7 mL), $K_2S_2O_8$ (0.044 g), and H_2O (102 mL). Microspheres with number average diameter $\bar{D}_n = 0.44$ μm and diameter polydispersity factor $\bar{D}_v/\bar{D}_n = 1.008$ ($\bar{D}_v$ denotes the volume average diameter) were obtained. The fraction of polyacrolein in the surface layer of microspheres was $f_A = 0.63$. Detailed description of the synthesis and characterization of microspheres is given in ref. [35].

Human serum albumin (HSA) (Sigma, Cohn fraction V) was used as received.

The surface of quartz slides was modified with a solution of γ-aminopropyltriethoxysilane in toluene (concentrations from 2 to 8%) according to the method similar to developed by Weetal [37]. The modification was carried out at room temperature for 18 h. Modified slides were washed with ethanol and with water. Before immobilization of the P(SA) microspheres slides were incubated during 5 h in phosphate buffer saline (PBS, pH = 7.4). Immobilization of microspheres was carried out by incubating quartz slides with the P(SA) microspheres (concentration 2 mg/mL) in PBS during 5 or 8 h. Thereafter, the slides with immobilized microspheres were washed with water. The HSA was attached to P(SA) particles by incubating a solution of HSA ([HSA] = 0.3 mg/mL) and quartz slides with the covalently immobilized microspheres. Incubation with HSA was carried out in PBS for 3 h. Finally, slides were washed with water, dried at room temperature at air for 24 h.

The surface of immobilized microspheres was observed using a Nanoscope III AFM instrument (Digital Instruments) in the tapping mode. None of the pictures presented

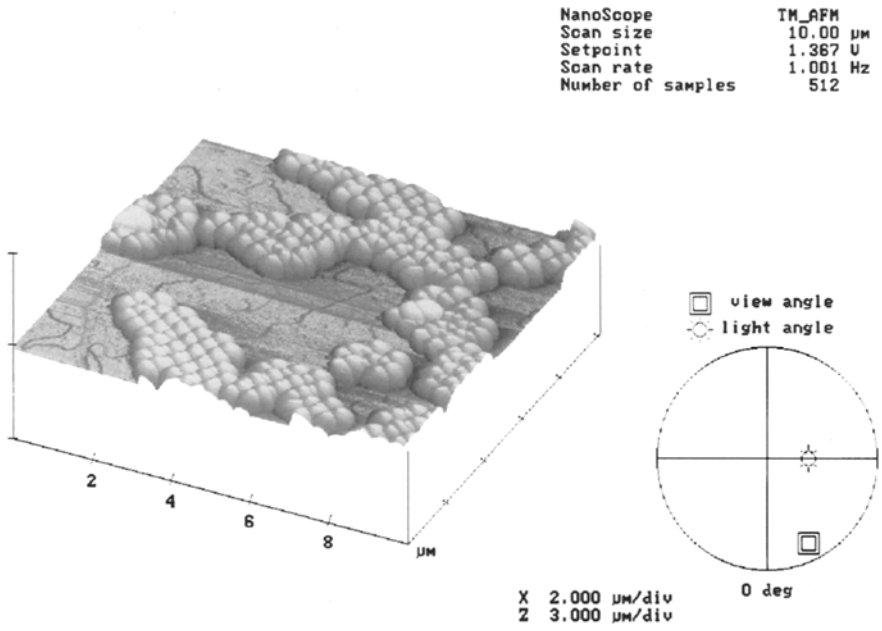

Fig. 1 Assembly of P(SA) microspheres immobilized on quartz

and analyzed in this paper was treated with any computer filtering procedures. The only computer manipulations involved choosing the required view angle and in some instances zooming.

Results and discussion

A typical assembly of P(SA) microspheres, without HSA, immobilized on quartz is shown in Fig. 1. Particles are arranged in clusters one particle thick. The partial coalescence of microspheres can be clearly seen. Apparently, in the case of the P(SA) microspheres, which according to our earlier findings [35] have a core-shell structure with a core rich in polystyrene and a shell enriched in polyacrolein, the segmental mobility allows for the interdiffusion of polyacrolein macromolecules in the surface layers of adjacent particles. A more detailed picture of the fragment of the surface of a typical P(SA) microsphere is shown in Fig. 2a. The curvature of the microsphere is clearly evident. The lines on the surface parallel to the x-axis represent the traces left by the tip and cannot be considered as a genuine characteristic features of the microsphere. From Fig. 2b it is evident that after attachment of HSA the AFM picture of the surface of microsphere becomes different. The lines left on the surface of the bare particles by the tip are absent. The surface illustrated in Fig. 2b is covered with a regular pattern of small "hills" and "depressions" with diffuse edges. Thus, it becomes clear that AFM allows to discriminate between the bare surfaces of the P(SA) microspheres and the surfaces covered with HSA. However, we think that such micrographs cannot be used as a basis for any conclusions on the arrangement of HSA macromolecules on the surface. We expected that we could obtain more information from the analysis of defects in the HSA layers on the surface of the particles.

The detailed inspection of many micrographs revealed that during drying the cracks and faults on the surface covered with HSA uncovered on some particles a multilayered structure of the attached protein and formed a very characteristic rift pattern. An example illustrating layers of HSA on the P(SA) microspheres is shown in Fig. 3. The cracked and displaced layers of HSA exposed the inner HSA layer covering microspheres. An example of a cross-section of microsphere with exposed HSA layers is shown in Fig. 4. On this and on similar micrographs it was possible to measure the thickness of the outer HSA layers. The measurements gave a value 8.6 ± 1 nm. In some instances we noticed the layer with thickness 15 ± 3 nm with edges suggesting that it is composed of two layers.

In several cases we found defects in the coverage of particles with HSA. For example, in Fig. 5 there is shown the picture of a surface covered only partially with HSA. On the part not covered with the protein the typical traces left by the tip can be seen. The cross-section of the border between areas without and with HSA is illustrated in Fig. 6. The defects in the coverage allowed us to measure the thickness of the first layer of HSA on the surface of the P(SA) microspheres. We found that the first layer of HSA macromolecules is 3.0 ± 0.2 nm thick.

It is interesting to compare the thickness of the HSA layers on the P(SA) microspheres with the dimensions of

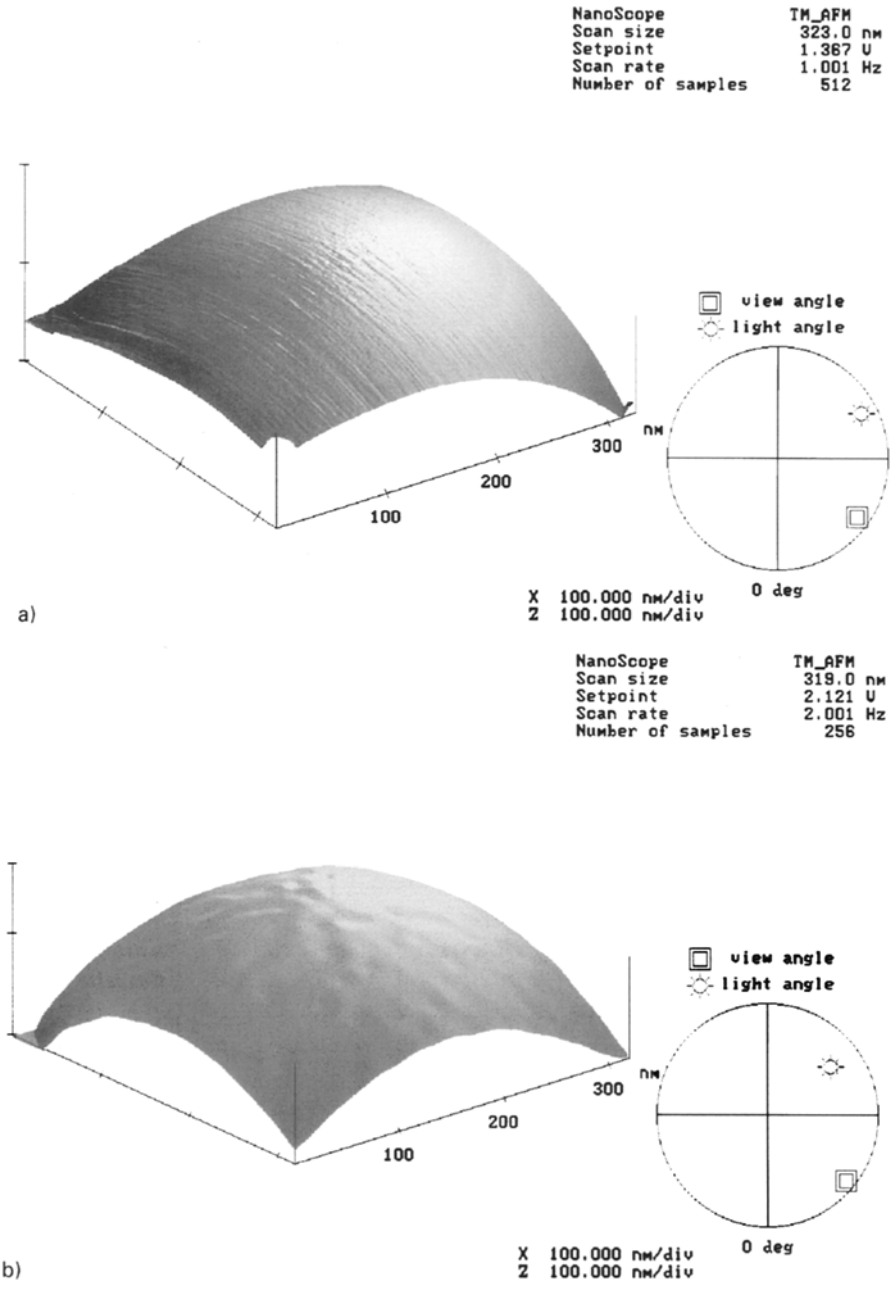

Fig. 2 a) Surface of a P(SA) microsphere. b) Surface of a P(SA) microsphere covered with HSA

the HSA macromolecules in the crystal form. As has been already mentioned, Carter et al. found that the three domains of the HSA macromolecule are arranged in the heart-like form with the sides 8.3 nm, 7.0 nm, and 8.2 nm, and a thickness of ca 3.0 nm [27]. It is worth noting that the thickness of the first layer of HSA on the P(SA) particles measured by AFM (3.0 ± 0.2 nm) is very close to the thickness of the HSA macromolecule determined by x-ray studies (3.0 nm). Moreover, the thickness of HSA in the outer layers determined in our work (8.6 ± 1 nm) is close to the long sides of the HSA macromolecule in the crystal form. Thus, the results of the AFM observations suggest that the HSA macromolecules in the first layer are arranged flatly on the P(SA) microspheres, maximizing their contact with the surface of particles. On the other hand, the HSA macromolecules in the subsequent layers (in the case of some particles up to three such outer layers were noticed) are arranged protruding from the surface. Such orientation maximizes the number of protein macromolecules covering each particle.

There are opinions that at pH = 7 macromolecules of HSA are adsorbed to the anionic and hydrophobic

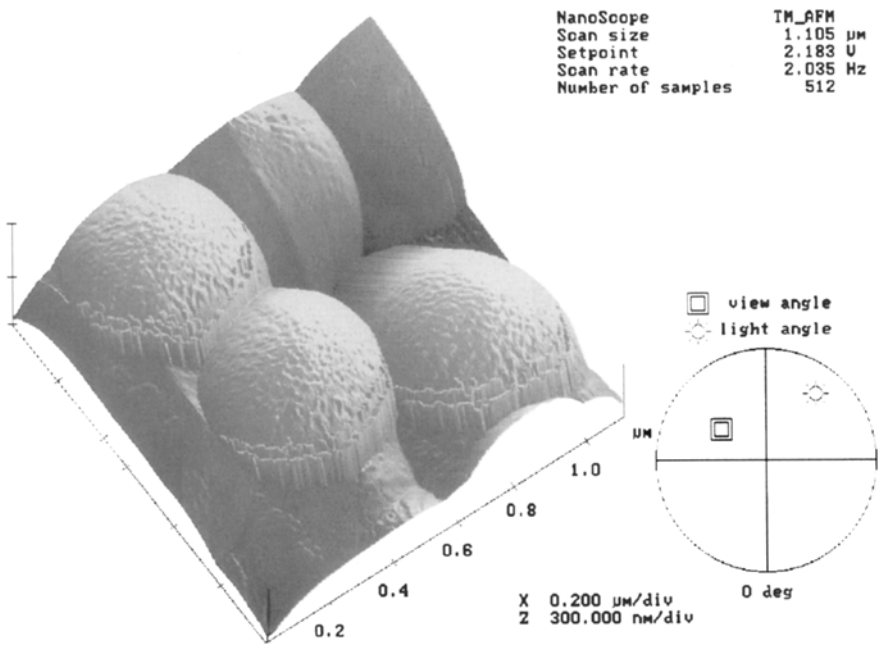

Fig. 3 Multilayered coverage of P(SA) microspheres with HSA

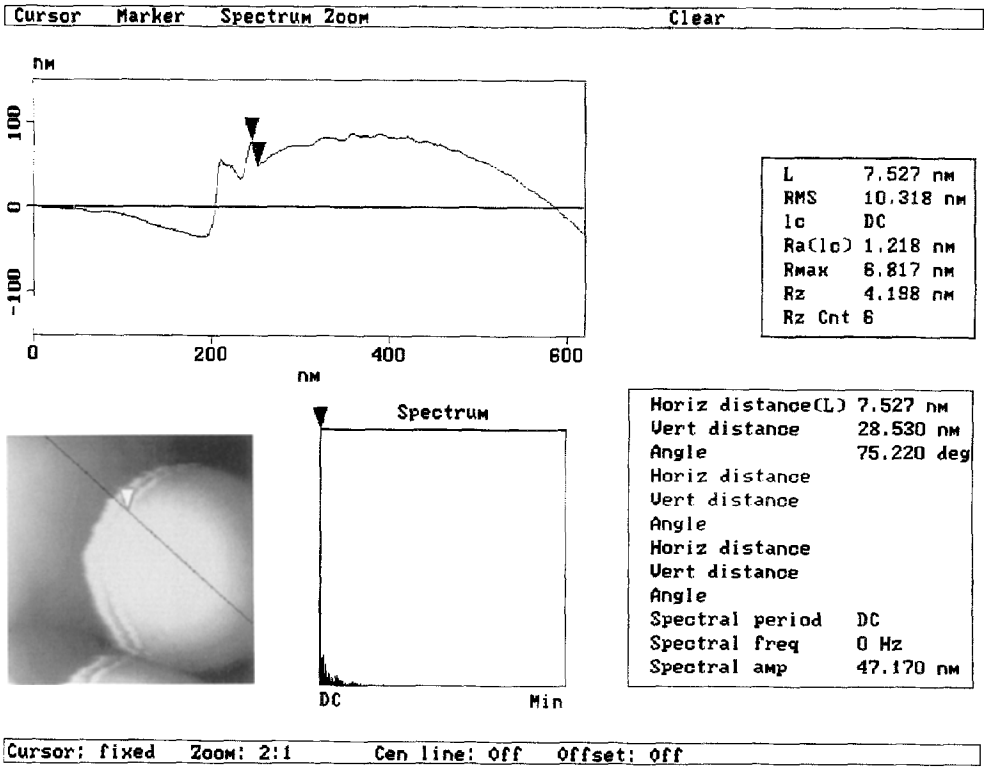

Fig. 4 Cross-section characterizing the multilayered coverage of a P(SA) microsphere with HSA. Horizontal distance between the pointers equals 7.53 nm

surfaces preferentially via the domain III, however, it has been pointed out that the thickness of the hydrodynamic radius of HSA on polystyrene equal to 4 nm conforms to the side-on adsorption involving all three domains [38].

In our earlier studies we found that the maximum surface concentration of HSA attached to the P(SA) microspheres equals 1.3 mg/m^2 [33]. This corresponds to $1.13 \cdot 10^4$ macromolecules on 1 μm^2. Data obtained from

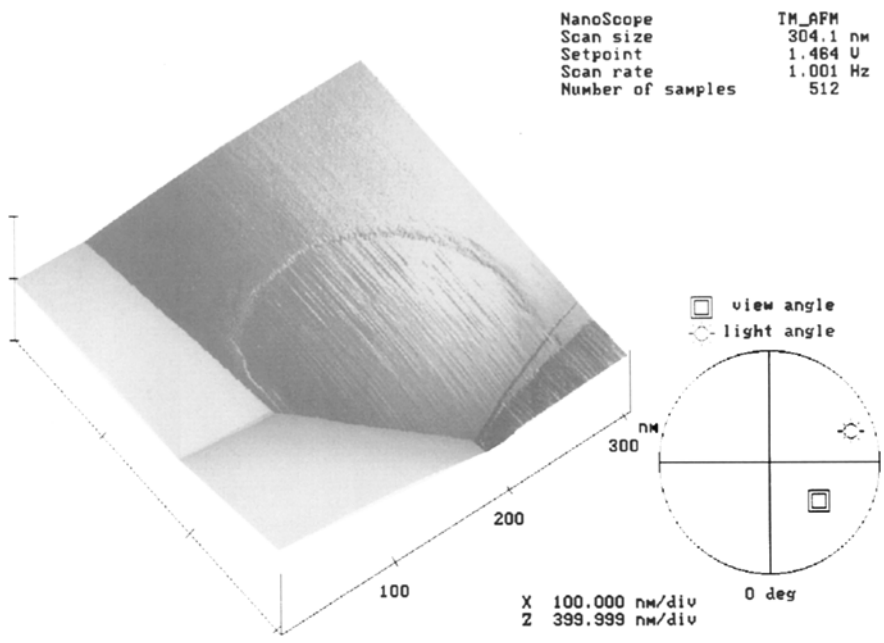

Fig. 5 AFM micrograph illustrating partial coverage of a P(SA) microsphere with HSA

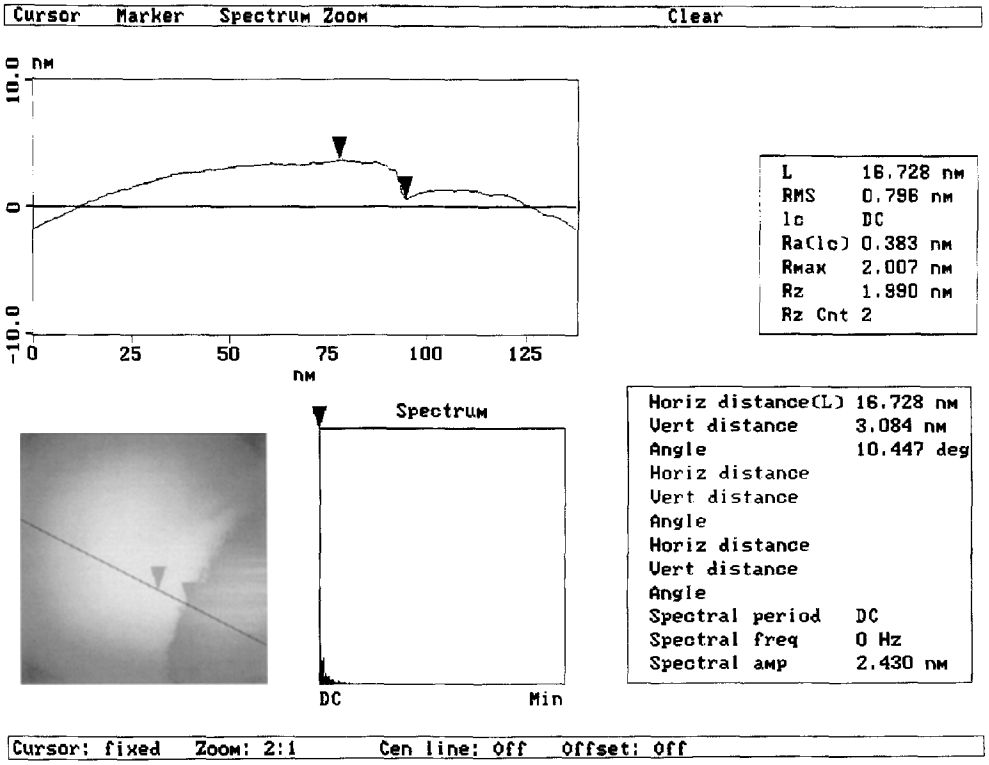

Fig. 6 Cross-section of the first layer of HSA covering a P(SA) microsphere. Vertical distance between the pointers equals 3.08 nm

x-ray studies indicated that the HSA crystals belong to the $P42_12$ space group with unit cell constants $a = b = 18.65$ nm and $c = 8.18$ nm [27]. However, assumption that the distance between macromolecules of HSA in the monolayer is the same as in the mentioned crystal (18.65 nm) would require the number of HSA macromolecules per 1 μm^2 to be $2.88 \cdot 10^3$, i.e., much lower than that determined from the above mentioned studies of

the immobilization of HSA on the P(SA) microspheres. Thus, it becomes evident that HSA molecules in monolayer on the P(SA) microspheres are packed more densely than in the HSA crystal. This is not surprising because it has been found that HSA crystals contain ca.77% of solvent molecules in the crystal channels with cross-section ca. 9.0 nm wide. Remembering that the cross-section of HSA macromolecule has the form of triangle with ca. 8 nm side, we can estimate that at the dense packing the number of HSA macromolecules with the side-on orientation equals $3.6 \cdot 10^4$ on 1 μm^2. The surface concentration of HSA macromolecules at dense packing is therefore about three times higher than that determined from the studies of attachment of HSA to the P(SA) microspheres ($1.13 \cdot 10^4$ macromolecules/μm^2) [33]. This difference might be related to an extensive denaturation of the HSA and/or to the loose distribution of macromolecules, or macromolecular clusters, on the surface of the P(SA) microspheres. The following reasoning suggests that the first possibility is more probable, however, we cannot exclude that also the second plays some role.

It is worth noting that surfaces covered with HSA monolayer are rather smooth (cf. Fig. 5) whereas in the case of microspheres with higher degree of the coverage with HSA "hills" and "depressions" are observed (cf. Fig. 4). We know that in the case of the P(SA) microspheres used in our experiments most of the attached HSA (ca. 90%) is immobilized covalently [33]. Apparently the protein macromolecules which are immobilized covalently evenly cover the surface of the microspheres whereas in the outer layers the adsorbed macromolecules retain some degree of lateral mobility and form clusters located from 20 to 30 nm from each other.

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