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HSA PARTICLE SIZE CHARACTERIZATION BY AFM

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Abstract. The atomic force microscopy (AFM) is a technique that has extensively been used to reveal details on surfaces using different scanning techniques. The human blood components, Human Serum Albumin (HSA) being one of them, as opposed to hard condensed matter samples, are soft and do not adhere well to surfaces, therefore imaging and analyzing soft condensed matter samples using AFM requires certain precautions and special care. This article presents details on using AFM for imaging and size characterization of the HSA. The results suggest that the HSA molecule has an average size of 36 nm, support the ellipsoid model for the molecule shape and indicate that on mica substrate the HSA molecule does not have a specific orientation.

Key words: atomic force microscopy, human serum albumin.

1. INTRODUCTION

The living cells interact with the environment or different stimuli. Investigating the cells in interaction is important in developing medication for the considerable number of diseases that affect the human body and affect the quality of life. Surface characteristics as surface energy, surface texture, hydrophobicity, or patterning at various length scales, surface charge, and chemical composition play crucial roles in dictating cell adhesion and growth [1].

Between many investigation techniques, imaging remains between the most frequently used and preferred. In order to keep the cells alive and functioning as normal as possible during their interaction with the stimuli, the imaging device must disturb the cell as less as possible. Optical microscopy appears to be the most suited for this purpose and the history of using this technique is quite extended. The optical microscopy has limitations though. Light diffraction limits resolution to about 0.2 microns, therefore optical microscopy is unsuited for measuring the submicron cells or serum components.

An alternative technique that can be used to visualize the submicron particle size is the Atomic Force Microscopy (AFM). Reference [2] reports using AFM even for nanoparticle sizing. Nevertheless, the preferred technique that is currently used to image nanometer sized objects is the Transmision Electron Microscopy (TEM) and a comparison of the TEM with the AFM results is presented in [3]. The TEM technique produces accurate results for nanoscale objects sizing but requires a particular sample preparation and using it in vacuum as a target for tens of Kevs electron beam. The size and shape measured after electron beam irradiation and in vacuum might be considerably different from the size and shape measured *in vivo*. For this reason the TEM technique is not widely used for blood serum components imaging. Moreover, the TEM technique presents systematic errors as well [3]. Another paper that presents results on using AFM on nanoparticle and nanoparticle aggregates sizing and a comparison of the sizing with Dynamic Light Scattering (DLS) results is [4]. DLS was not used in the work presented here because it provides information regarding the size but not regarding the shape of the particles in suspension.

HSA is another submicron object that has been extensively investigated. Albumin has an extended range of applications including research as a protein model, pharmaceutical, clinical practice, diagnostic reagents, solution in chemical analysis, even food chemistry. As nanotechnology is developing fast, effort is made to develop nanoplatforms for drug delivery. Reference [5] reports on fabricating HSA coated iron oxide nanoparticle with doxorubicin attached on the HSA coating. The doxorubicin-HSA coated iron oxide nanoparticles were found to have a striking tumor suppression effect that outperformed free doxorubicin [5]. Reference [6] states that HSA nanoparticles surface modified with tumor necrosis factor and transferrin, also containing doxorubicin, were designed and prepared and were found to have remarkable cytotoxic and apoptotic activities in all cancer cells examined.

As HSA has an increasing role in drug delivery engineered nanoparticles, an accurate knowledge regarding the size and shape is most useful. References [7] and [8] report on investigating the HSA size deposited on mica substrate using AFM. The work in [7] indicates a size for the HSA around 25-30 nm resulted from analyzing individual profiles, therefore using a relatively poor statistics. Reference [8] reports on clusters of molecules, on features that were attributed to individual molecules and on the AFM used in tapping mode to visualize the three main domains of the HSA molecule and to modify the distance between them, hence the size of the molecule. The work reported in this paper was carried on with the purpose of producing results regarding HSA size and shape with improved accuracy with the AFM technique using both an improved grain statistics and by extracting and analyzing individual profiles. Another purpose of the work reported here was to investigate the orientation of the HSA molecule deposited on freshly cleaved mica substrate.

2. MATERIALS AND METHOD

The sample that was studied using the AFM microscope was the HSA. The HSA is a single polypeptide with 585 amino acid residues containing 17 pairs of disulfide bridges and one free cysteine [9]. It is the most abundant protein in human blood plasma, is produced in the liver and comprises about half of the blood serum protein. The reference range for albumin concentrations in blood is 30 to 50 g/L. It has a serum half-life of approximately 20 days and a molecular mass of 67 kDa [10].

The sample preparation for imaging and characterizing HSA using AFM resembles very much the procedure presented in [7]. Small amount of HSA 200 g/L in 136.5 mM NaCl aqueous solution produced by Kedrion, a typical solution used in intravenous infusions, was further diluted in deionized water to reach 2 g/L. The reason for further dilution was to obtain a single layer of HSA macromolecules on the substrate after the final stage of sample preparation. A small drop of the 2 g/L HSA solution was deposited on a freshly cleaved mica substrate. It was stretched on the substrate using a sharp edge and allowed to evaporate for 6 hours at 20 ^{o}C in 45% humidity air.

The AFM is a scanning probe microscope and uses a flexible cantilever to measure the force between the tip and the sample or to describe a profile while maintaining the force or electric current between the tip and sample constant [11]. By scanning the sample line by line and using a calibration file for each scanner a topography image of the surface is reconstructed and can be used in particle image and sizing.

The AFM that was used in the work reported here is an Agilent 5500 type. The scanning was carried on in tapping mode. A very soft tip, with the spring constant equal to 5 N/m was used at low force amplitude.

Sample preparation is very important in order to avoid artifacts. The samples must be thin enough to have, whenever this is possible, only one layer of the objects under investigation, whether they are micron sized cells or nanometer sized particles. Moreover, the objects to be imaged must stick well to the substrate and remain in the same position during scanning, otherwise they will be moved by the tip of the cantilever, producing artifacts and unrealistic images. More details on the scanning using the AGILENT 5500 AFM are presented in [4] and [7].

First a relatively large area, large when compared with the size of the object being investigated, 2 μm × 2 μm was scanned, followed by a bigger resolution scan of a smaller area, 0.5 μm × 0.5 μm. A 3D rendering type image is obtained and can be used to asses in a half-quantitative manner the dimension of the HSA molecule.

As described in detail in [4] and [7] a 2 g/L volume ratio concentration is quite big and there are many albumin molecules on the area that was imaged,

therefore assessing the protein dimension from a 3D image is not accurate. A better way to assess the particle dimension on a surface is to extract vertical profiles [4], [7] and to use them in measuring the distance between the baseline and the top of the peaks.

Moreover, the cantilever tip wears out during scanning, therefore exhibiting a bigger tip radius. Even the sharp new tips have a tip diameter around 40 nm and are used to scan details of comparable size or smaller. The last figure of [4] illustrates the tip during scanning over a nanoparticle and clearly explains that measuring the horizontal difference between two consecutive valleys on a profile produces a value that is systematically bigger that the diameter of the nanoparticle while measuring the distance between the base line and the top of the peak on a profile produces more accurate diameter results. This is true especially because the radius of the tip increases as the scanning process continues and the tip wears out.

3. RESULTS AND DISCUSSION

As described in the previous section a good resolution scan (512×512) was achieved for a surface of 0.5 μm × 0.5 μm. Once the acquisition was completed, a simple image processing was carried on. First a three point leveling was applied, to correct the frequently occurring small tilt of the substrate. The horizontal artifact lines were removed using the scar removal procedure. A 3D rendering type of the surface topography is presented in Fig. 1.

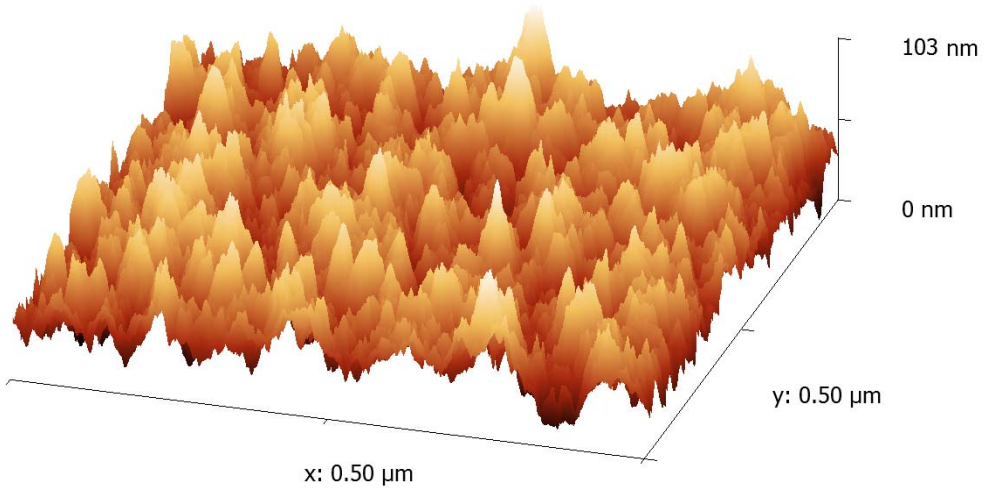

Fig. 1 – A 3D rendering type of a 0.5 μm × 0.5 μm area of HSA on freshly cleaved mica substrate.

Examining Fig. 1 we notice from the beginning that the peaks on the surface are sharper than in the 3D image presented in [7] simply because a new tip was used while in the work presented in [7] the tip had already been used for several hours of scanning. Different profiles were extracted from the topography image in Fig. 1 and two of them are presented in Figs. 2 and 3. The *Z* difference between two points in the vertical profile was considered in assessing the size of the HSA molecule, rather than the *x* or *y* difference, as previously explained.

Examining Figs. 1 and 2 we notice that the difference between the base line, (that was set to 10 nm) and the top of the albumin molecules in profiles is between 30 and 60 nm.

The grain statistics provides more accurate information regarding the objects that cover the substrate surface. The grain identification and statistics was carried on using Gwyddion 2.6 software. The grain size distribution is presented in Fig. 4. Examining Fig. 4 and the results of the statistical calculation performed on the scan we notice that the distribution has an average of 46 nm and that the maximum size is 103 nm. If we draw a line at half-maximum of the distribution we notice that it corresponds to 29 nm and 59 nm.

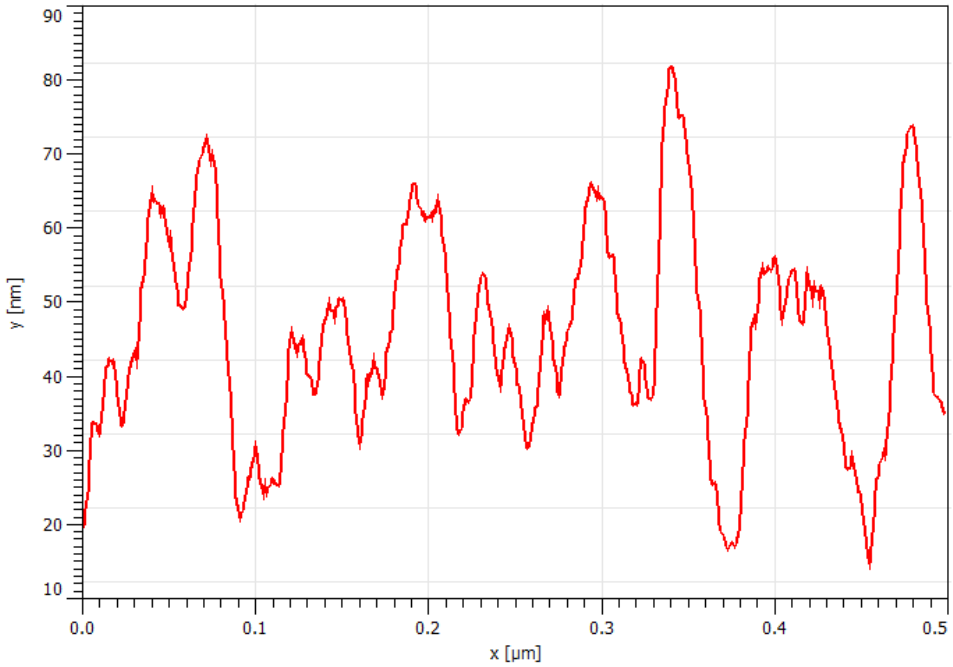

Fig. 2 – A profile extracted from the scanned area presented in Fig. 1.

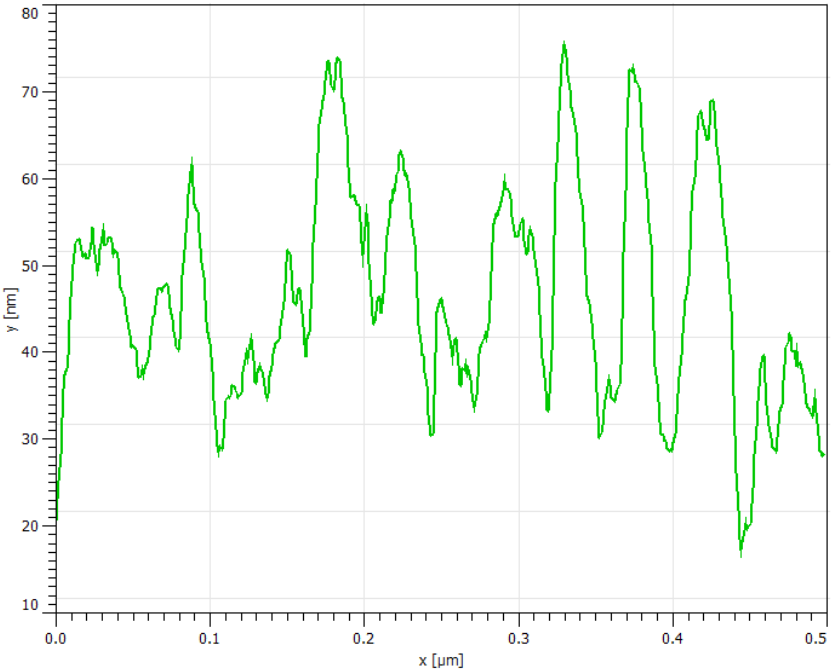

Fig. 3 – Another profile extracted from the scanned area presented in Fig. 1.

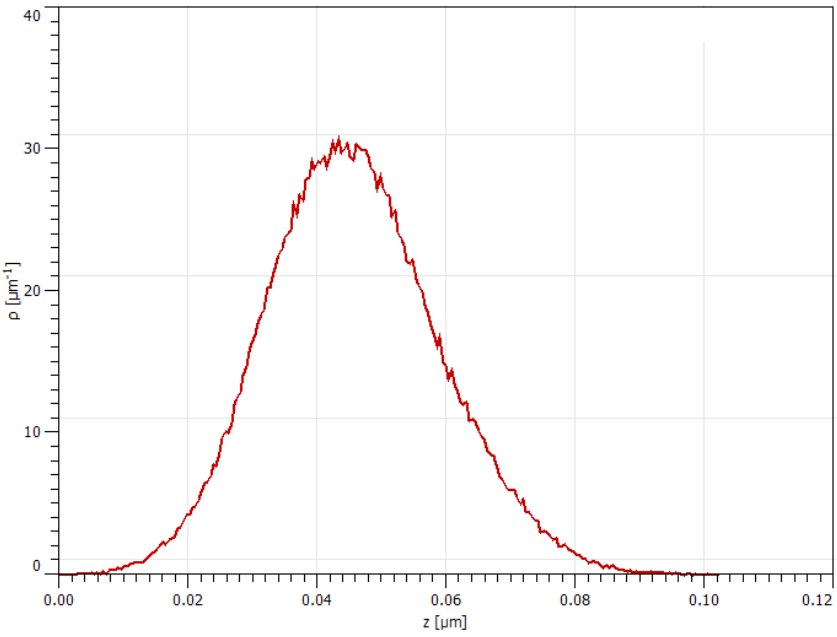

Fig. 4 – The grain size distribution of HSA on mica substrate.

Figs. 2 and 3 and the other extracted profiles reveal that the lowest cantilever tip position during scanning was around 10 nm. The vertical differences that lead to the object dimension should therefore be the difference between the top of the peak and of the lowest cantilever position. This indicates an average size of 36 nm.

The literature reports different sizes for the HSA molecule. Using the dynamic light scattering technique (DLS) [12] the albumin globule was found to have the most compact configuration (Stokes diameter 59–62 Å) at physiological pH 7.4. The changes in pH, both an increase to 8.0 and a decrease to 5.4, produced a growth of the HSA globule size to 72–81 Å [12], [13]. At acidic shift of pH an additional peak arises in the DLS correlation spectra produced by the light scattering on the structures with the Stokes diameters of 29–37 Å. This result is consistent with the sizes of the albumin subdomains [12, 13].

A different investigation of HSA in 150 mM NaCl aqueous solutions was carried on using small-angle neutron scattering [14]. The radius of gyration of albumin molecule was found to be 27.4 ± 0.35 Å. A compact sphere model leads to a smaller radius of gyration, while in the cigar-shaped model for the molecule, about 136 Å long, would have a larger radius of gyration [14]. Other suggested shapes for the HSA were a prolate ellipsoid less than 110 Å long or an oblate ellipsoid about 85 Å [14].

The results we obtained during our AFM size analysis are comparable, within the systematic unavoidable errors described briefly in this article and in details in [4], with the neutron scattering results [14] The DLS results [12, 13] indicate a size of the HSA molecule considerably bigger that our results. This difference is quite normal as the DLS technique provides a hydrodynamic diameter, slightly bigger than the physical diameter measured on dry samples. The HSA was also found to exhibit crystalline phase [15]. The preliminary diffraction studies they conducted revealed that the crystals belonged to the tetragonal space group $P42_12$ with cell dimensions a = 187.1 Å, c = 80.5 Å [15].

The results of another AFM study of HSA deposited not on mica but on highly oriented pyrolytic graphite [16] revealed two different shapes: circular with a diameter of 25 ± 3 nm and oblong with the sizes of 35 ± 5 nm × 24 ± 2 nm with the height in the range 2.5 ± 0.3 nm. These results are comparable with the values reported in this article for HSA molecule size. The work in [16] suggests that on highly oriented pyrolytic graphite the HSA molecule has a well defined orientation, with the big dimension along the surface. The size distribution presented in Fig. 4 is smooth, without one sharp peak, which would correspond to a specific orientation or without two sharp peaks, which would correspond to two possible orientations of the ellipsoid shape molecule. The relatively wide size distribution, with 30 nm width at half maximum, as revealed by Fig. 4, supports the ellipsoid model rather than the spherical model, which would have produced a narrower distribution, if the objects under investigation were spherical. Moreover, this smooth size distribution in Fig. 4 suggests that the HSA on mica substrate does not have a specific orientation.

4. CONCLUSION

The results we found during our AFM size analysis are comparable with the neutron scattering results on HSA in saline solution. The HSA size we found is smaller than the DLS size reported in the literature.

The differences of the HSA size in the AFM results from the DLS and neutron scattering results can be explained both by the differences in the methods that were used and by possible crystallization effects. The AFM samples were dried in air, thus enabling crystallization, while both the neutron scattering and the DLS measurements were performed on liquid samples under various pH values.

Nevertheless, the size of the HSA molecule we found using an AFM analysis is consistent with the ellipsoid model for the molecule and with the AFM results on HSA deposited on graphite substrate. Moreover, our results suggest that the HSA capsule deposited on mica substrate does not have a specific orientation, while on highly oriented pyrolytic graphite the HSA molecule has a well defined orientation, with the big dimension along the surface, as reported in the literature.

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