CHARACTERIZATION OF SELF-ASSEMBLED PROTEIN THIN FILM ON FLAT AND NON-FLAT SUBSTRATE

CARACTERIZAÇÃO DE FILMES FINOS DE PROTEÍNA SOBRE SUBSTRATO PLANO E NÃO PLANO

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ABSTRACT

In this study we have characterized the surfaces of lysozyme globular protein films self-assembled on glassy substrate. Flat slides and cylindrical glass fibers were used as solid supports for protein (concentration 10^{-4} M) immobilization and the surfaces scanned with atomic force microscopy (AFM) along the deposition time. AFM data allowed following the surface roughness reduction as the film was formed. The deposition features were quite similar for both substrates, whereas on the flat surface a more regular deposition was observed. The thickness of grafted layers was determined from differences between deposited and non-deposited regions and measured, after 10 min immersion, as 7.6 ± 1.4 for flat and 10.4 ± 2.6 nm for cylindrical surface, suggesting by comparison with the protein molecule dimensions, that the adsorption should be a multilayer process under the experimental conditions used in this work.

Keywords: Self-Assembly, Protein Films, Protein Immobilization, Surface Analysis, AFM.

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RESUMO

No presente estudo caracterizamos a superfície de filmes automontados da proteína globular lisozima sobre substrato vítreo. Lâminas plana e fi[1]bras de formato cilíndrico foram empregadas como substratos para imobilização. Solução protéica na concentração de 10^{-4} M foi utilizada e os filmes em sua forma final analisados por AFM (microscopia de força atômica). Os dados possibilitam acompanhar a redução da rugosidade conforme o filme é formado. Os depósitos, contudo, são similares para ambos os substratos sendo a superfície plana a que proporcionou uma deposição mais regular. A espessura dos filmes formados, após 10 min de imersão, foi determinada a partir da diferença entre regiões depositadas e não-depositadas e estabelecido como 7,6 ± 1,4 para o substrato plano e 10,4 ± 2,6 nm para a superfície cilíndrica, sugerindo por comparação com as dimensões de uma molécula da proteína que a adsorção, nas condições experimentais aqui empregadas, ocorre de forma multicamadas.

Palavras-chaves: Automontagem, Filmes de Proteína, Imobilização, Análise de Superfície, AFM.

INTRODUCTION

The self-assembly (SA), or as recently referred layer-by-layer (LL) technique, has been largely used for the fabrication of ultra-thin films and multilayers systems of biomolecules in a controlled fashion (WHITESIDES, 1995; PATERNO et al., 2001). This technique, in conception, refers to a spontaneous attachment that takes place during the contact of molecules dispersed in a fluid phase with a solid support (BISHOP & NUZZO, 1996; LVOV, 2000), or as defined by LINDOY & ATKINSON, 2001, a process by which a supramolecular species forms spontaneously from its components and are held together by a range of relatively weak intermolecular interaction.

A large number of polymers and molecules which present spatial distribution of charges or amphiphilic structures are capable to be deposited in an ordered manner, under suitable solvent conditions, on appropriated substratum. In particular macromolecules, such as proteins and polysaccharides, form nanostructured films owing to its polar characteristics and charges development in liquid medium. One important feature in this mechanism is that the process is rather dependent of the solution acidic level, i.e., at specific pHs charges can be developed along the polymeric chain and preferential orientation takes place between the molecules and the substratum. The hydrophobic groups of the polymer chain will be associated with the surface hydrophobic groups of the substratum and vice-versa (FENDER, 1996). That is an important characteristic that can define SA films potential applications.

The globular protein lysozyme, also known as muramidase, is a natural thermally stable enzyme found in colostrum, hen egg whites and human nasal mucus and tears. It is characterized by multiples and complex structural groups defining spatial hydrophobic and hydrophilic regions, with predominant positive charges as models constructed by BLAKE et al., (1995), and KAYUSHINA et al., (1996). SA lysozyme films have been tested as filtration media (ASSIS & CLARO, 2003) and as heavy metals (CHERIAN, et al., 2003) and bacteria sensors (HUANG et al., 2003), amongst several possibilities.

The substratum suitable for the lysozyme spontaneous bonding has to present high density of negative superficial charge, such as metal or glass, attracting the oppositely charged portion of the molecules. In glassy materials, the hydroxyl groups on the surface provide functionality that react with hydrolysable groups of protein structure (ASSIS & CLARO, 1999; SENGUPTA, 1999). According to JIA & LIU, (2005) the lysozyme deposition pattern follows the same behavior as observed to amphiphilic molecules.

The format of the solid substratum also plays important role on the formation of the film. ULMAN, (1991), states that the deposition in horizontal flat surfaces is favorable to a more

stable film assembly, although it presents the disadvantage of not being possible to control the molecular array uniformity. According to DE-BACKER & BARON, (1993), immobilization on porous glass media is usually irregular and it changes diffusivity on permeable medium. However, AVNIR et al., (1994) showed that porous glass surface can be advantageous in effective trapping of enzymes although most of the surface available for activity is internal. Conversely, good fixation of enzymes is attained on non-porous glass beads making use of crosslinking covalent agents (IKEDIOBI, et al., 1998).

The immobilization of enzymes on non-flat media, such as porous ceramics or fibrous support, has technological importance for configuring bioreactor or biofiltration medium of which some liquid permeation is mandatory. In this work we use the atomic force microscopy (AFM) for characterizing lysozyme self-assembly film formation on cylindrical fiber glass, comparing morphology with similar deposition on flat slides.

MATERIAL AND METHODS

Commercially available glass fibers of 70-140 µm diameter and glass slides (5mm x 10mm x 2mm optical glass plates) were used as supports. Both materials underwent chemical functionalization treatment, named 'piranha' method, that consists of a series of surface cleaning procedures in warm acid solutions using ultra-sonic baths, as described elsewhere (KERN, 1993). This treatment enhances the glass negative surface charges and the corresponding hydrophilicity index.

An aqueous solution of lysozyme from hen egg-white protein (Sigma) was prepared (as delivered) at a concentration of 10^{-4} M, using ultrapure deionised water, and the solution adjusted to pH ~ 6.2, using phosphate buffer (Na_2HPO_4).

The supports were kept immersed in protein solution and samples randomly removed after 5, 8, 10 and 12 min immersion and then rinsed in distilled water. Figure 1 schematically illustrates the experimental set up. AFM images were acquired in non-contact mode (TopoMetrix Discover System) and the images processed by a TOPOSPM software. Two random areas of 1 ìm x 1 ìm were scanned on each support. A total of 4 flat and 4 cylindrical samples were analyzed resulting in a total of 8 areas scanned in each condition. The film thickness was measured as the average differences on non-deposited and deposited terraces after 10 min immersion, using a methodology previously described by ASSIS & SILVA, (2003).

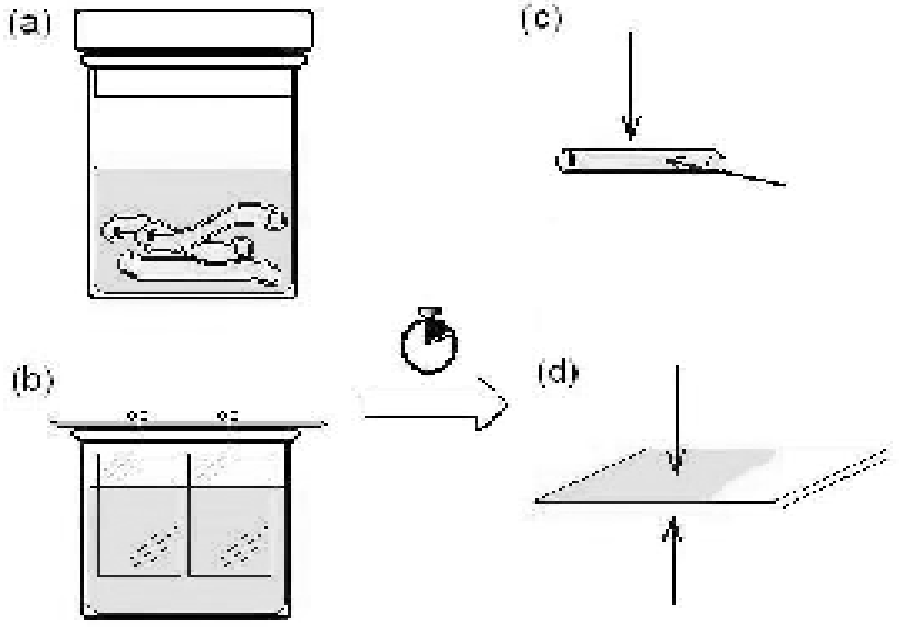


Figure 1 - Illustration of protein deposition process and faces analysis. In (a) and (b) the fiber and glass slides are separately immersed in the precursor solution and samples withdrawn at 5, 8, 10 and 12 min. Each fiber had two regions randomly scanned (c) and the slides had the both side also examined by AFM (d).

RESULTS AND DISCUSSION

Protein adsorption on solid surfaces is frequently modeled as the formation of a two-dimensional, ordered array positional parallel to the surface. The stability and homogeneity of deposition is stated to depend on the surface density of OH groups (INOUE et al., 1998), where the adsorption and desorption is determined by competition between attractive interaction and non-specific repulsion (SCHWARTZ et al., 1992; LEE et al., 2001). The film formation depends on the volume of protein molecules near the surface (FIGUEIREDO et al., 2005) and on the uniformity of initial sites occupation. The final film feature is than ruled by spontaneous packing-induced reconfiguration of individual adsorbed molecules on the solid surface (ROBESON & TILTON, 1996).

In the AFM morphological features are defined by x, y, and z coordinates, which indicate the relative height (z) of the cantilever tip at each x and y planar location. The surfaces irregularities can be expressed by the RMS (root-mean-square) roughness describing the standard deviation of an entire distribution of z-values for each scanned area as:

$$\sigma_{rms} = \left[(1/N) \sum_{i=1}^{N} (z_i - z_{av})^2 \right]^{1/2} \quad (1)$$

where z_i is the height of the i_{th} data point and z_{av} is the average value of z_i over all i's.

The RMS roughnesses acquired on surfaces randomly drawn from the solution resulted in the data summarized on table 1. For a better visualization of roughness data is plotted in Figure 2.

Table 1. AFM roughness (RMS) data for slide and fibers supporters as a function of lysozyme deposition time.

Sample	Deposition Time (min)	Average RMS roughness (nm)	Standard deviation*
Fibers	0	105.0	± 7.7
	5	58.4	± 3.7
	8	40.0	± 4.6
	10	37.3	± 6.5
	12	33.6	± 3.0
Slides	0	80.7	± 6.7
	5	18.1	± 5.5
	8	15.0	± 7.0
	10	10.7	± 5.4
	12	9.8	± 3.2

*performed over 8 scanned area.

From the starting uncoated surface (0 min) it follows, for both format of substrate, a roughness reduction as the deposition proceeds, which could be interpreted as the surface being recovering by lysozyme molecules, forming a continuous smooth film. Slides were found to be initially less rough than fibers and no significant differences in average roughness were found on fibers faces. Both materials presented similar rates of roughness reduction as protein deposition proceeds, in agreement to results found by BORATO et al., (1997), who reported, by ultra-violet monitoring, that lysozyme film formation is a first-order process with homogeneous distribution over surface after 8-10 minutes in precursor solution immersion.

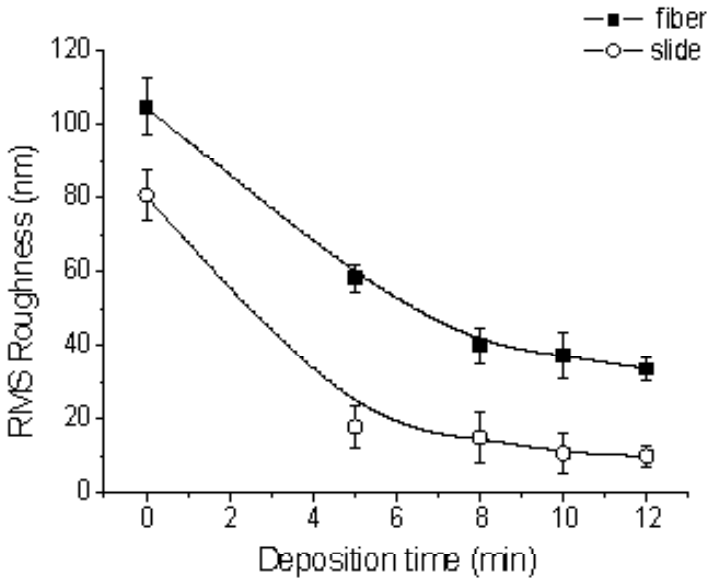
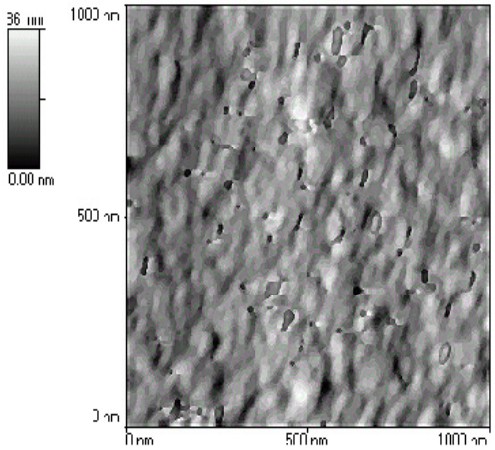


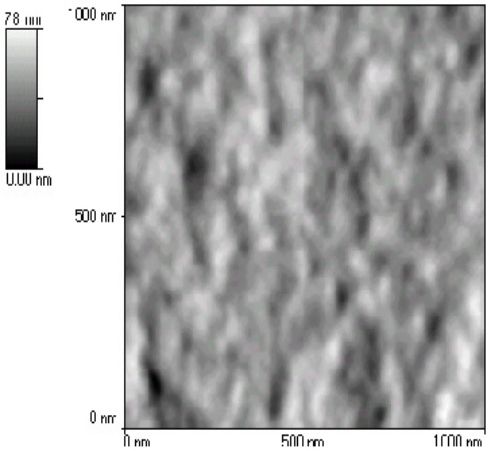
Figure 2 - RMS roughness as a function of deposition time.

The surface smoothing as enzyme adsorption takes place can be visually evaluated by means of qualitative information obtained from AFM images. As sequence shown in Figure 3 (for fibers scanning), it is possible, based on the appearance of the deposited film, to assess the overall reduction in roughness intensity. After a 12-min immersion, excess formation of lysozyme can be observed, which is probably a result from clusters deposition due to molecules aggregation while these are still in solution. Similar visual sequence was observed for flat substrate.

(a)



(b)



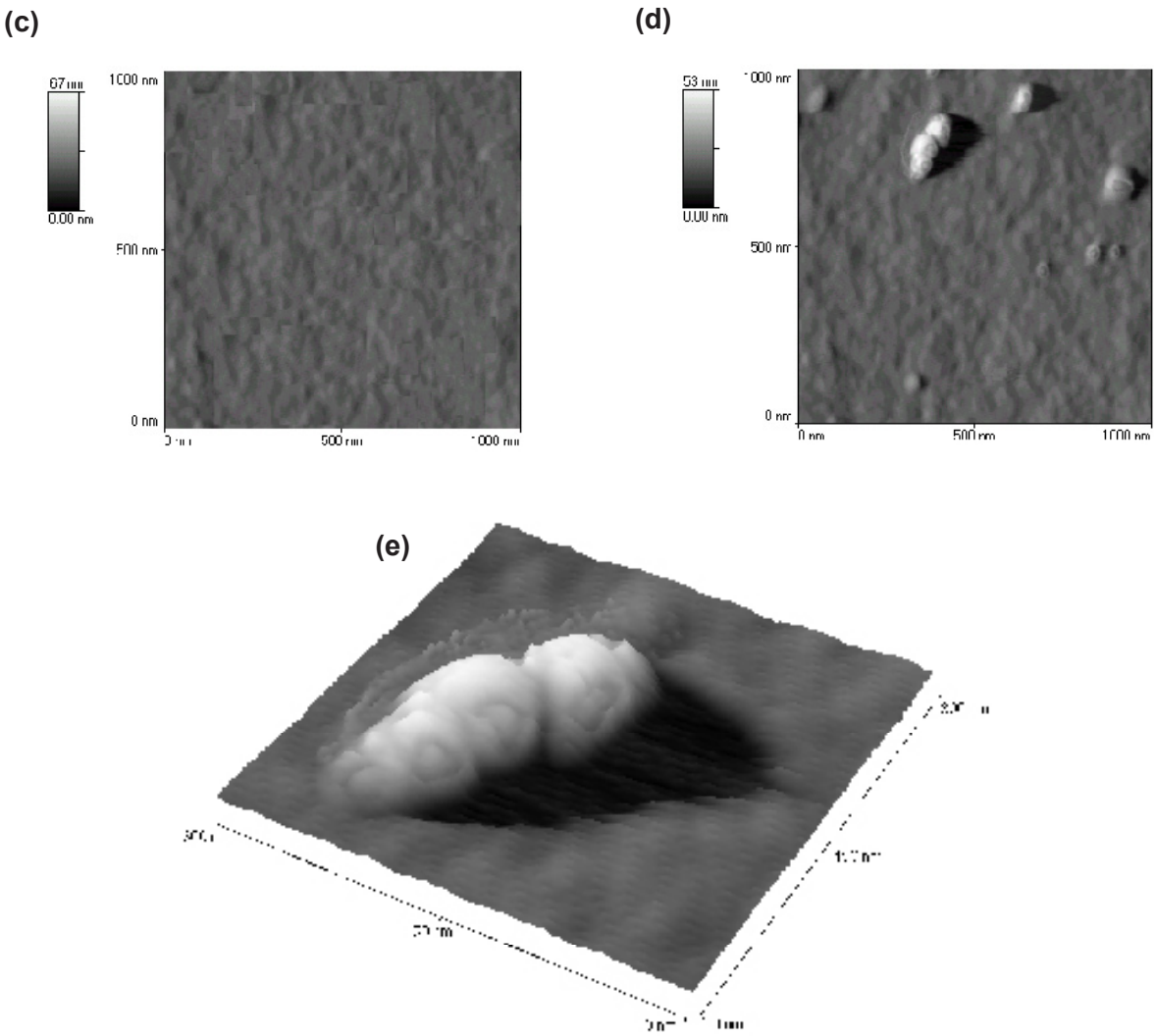


Figure 3 - AFM aspects of the scanned surfaces where changes in topographical features can be observed: in (a) as-hydrophilic fiber, (b) 5-min surfactant immersion (c) 10-min immersion and (d) 12-min immersion. Ridges observed after 12-min indicate excess of deposited protein, as zoomed in (e).

Thicknesses of formed layer after 10 min deposition were measured as 7.6 ± 1.4 for flat surface and 10.4 ± 2.6 nm for fibers which are larger than the size of lysozyme molecule indicating that the adsorption must be multilayer.

As lysozyme has an ellipsoidal shape with short axis of about 3.0 nm and a long axis of about 4.5 nm (LU et al., 1998), it can be assumed that a monolayer will have the thickness equivalent to one of the axial lengths. Nevertheless, thickness resultant of self-assembly is a quite controversial matter, for instance, POSTEL et al., (2003) measured thickness as between 2.5 and 3.0 nm on lyzozyme self-assembled monlayers. On the other hand, deposition as thick as 12 nm was found by SU et al., (2000), while analyzing monolayers of lysozyme on silicon oxide surface, leading them to conclude that the amount adsorbed is time-dependent, and recently, LUBARSKY et al., (2007) strongly states that thickness of protein films are dependent mainly on the solution concentration only.

Anyway, many of the physical properties and activity of mono or multilayer films depend on the film thickness (BLANK, 1981) and our results indicate that despite the similarities between deposition on flat and cylindrical surface, under AFM analysis, flat slides appear to result in a more uniform deposition which can reflect on

activity or response signal when applied in sensor or biobased devices.

CONCLUSION

The immobilization of lysozyme on chemically cleaned glass surfaces resulted in a dense deposition on which the initial roughness is reduced as more material is sequentially attached to the solid surface. Our results indicate that the initial surface roughness has greater effect on deposition features than the support format (flat or cylindrical); i.e., despite both materials presented similar rates of roughness, the initial roughness seems to be determinant for the process. On both supporters however, the measured deposition thickness are superior to an expected monolayer suggesting that the adsorption should be multilayer. Excess of deposited material was also observed at 12 min immersion. The results from this study, although semiquantitative, provide important insight into how initial roughness or support morphology can influence the formation of protein film formation via self-assembly technique.

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