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Protein Confinement in the Anodic Porous Alumina Microarray

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Abstract

Alternative approaches to solve the complexity of protein function in the proteomic field required new arrays in nano-micro-scale dimension. Anodic Porous Alumina (APA), a geometrically ordered inorganic oxide, grown from ultrapure aluminum by anodic oxidation, was used in this work as an ordered array for the confinement of protein molecules by spin-coating technique. This micro- or nanostructured material can be used in different ways, both for the traditional fluorescence approach and for novel label-free methods. The aim of the present study is the proof of principle that the protein molecules can enter the APA micropores, as shown by fluorescein-labeled lysozyme confined into the APA template. Fluorescence intensity measurement as well as Raman Spectroscopy confirm that protein was incorporated into the APA micropores.

Keywords

Anodic porous alumina, Fluorescence, Lysozyme, Spin coating

Introduction

Anodic porous alumina is a well-ordered inorganic aluminum oxide with hydrophilic negative surface charge and density of 1.2 g/cm^2. In recent years, APA has attracted significant interest for sensor [1-2], photonic devices [3] and protein crystals growth [4,5] due to the specific characteristics of its ordered multi-cavities array [6]. The fast development of the proteomic field and the complexity protein-protein and proteins-small molecules interaction require new advanced protein arrays in micro and nanoscale dimension. The high aspect ratio (depth/width ratio) of the pores makes APA a natural waveguide for any fluorescent molecule present on the bottom of the pores, avoiding crosstalk of many point-light sources too close. Moreover, APA offers a platform to investigate the protein-protein complementary interactions both with fluorescence and label-free approach [7] Tuneable pore size makes it possible to capture label free ligands, allowing to realized functionalized protein array for diagnostic of different type of diseases, including cancer. APA offers array of ordered cavities or pores (~10^8 pores/cm^2) for surface area, which can be used for the confinements of a wide range of proteins. Periodic cavities and pillars can be used for oligonucleotides [8], neuron growth [9] and protein confinements [10]. Anodic porous alumina was synthetized by electrochemical route for the first time by Masuda et al. [9], during the year 1995 and his electrochemical-processes it hasn't essentially changed during the last ten years. Several electrolytic solutions for the APA electrochemical synthesis, such as Phosphoric, Oxalic and Selenic acids are used to obtain a three-dimensional pore within ranging of 10 to 500 nm. AFM topography images can characterize

APA surface. Parametric conditions in electrochemistry are used to drive the phenomenon in particular working electrode nature, temperature, control-electrodes, electrode distance and electrolytes.

The currently confinement by spin coating technique can overcome such problems as bubbles air, capillary forces or sample heterogeneity, with no restriction to biological samples. The effect of confinement into the pores and the molecule's delectability into the nano channels were studied with use of Rhodamine B [11]. Functionalizing of APA surface can be done before spot deposition by spin-coating technique or by solution casting [12]. Several methods have been used to confine or impregnate dyes in APA cavities [13]. Spectroscopic investigations are of use for in the confinement studies also of dye molecules [14-17]. The present work aimed to confirm that APA surface able to host macromolecules in to its pores. For this reason, the images of fluorescence of labelled lysozyme confined onto APA surface was recorded by Charge Couple Device (CCD) camera. Carboxyfluorescein succinimidyl ester (CFSE) was choosing due to high fluorescence yield, distinct ionization forms and remarkable optical properties, conserved also when in contact with APA [18]. Due to its high solubility, it could be also used as a marker for dynamic liquid flow investigation. CFSE is an excellent reagent for amine modification since the fluorescent amide products formed are very stable. CFSE has high reactivity with aliphatic amines, low reactivity with aromatic amines, including tyrosine. CFSE has a wavelength excitation maximum of 492 nm and emission maximum of 517 nm, labeled protein can be detected with any instrument or filter set compatible with fluorescein detection. In our hands, CFSE-labelled lysozyme was already used for Langmuir-Blodgett protein template preparation [19]. Raman spectroscopy was used to characterize the conformation of CFSE–labelled lysozyme on APA surface. Obtained results can be potentially utilized for confinement of any other protein and developments of new protein microarrays for protein-protein interactions studies.

Material and Methods

Aluminium foils was supplied by Goodfellow (USA), thickness 3 mm and purity 5N (99.999%). Lysozyme was purchased from Sigma Aldrich with a purity of 99.99 % and stored at 4 °C. Carboxyfluorescein succinimidyl ester (CFSE) (Molecular Weight of 376.28 g/mole; solubility in water up to 500 g/l at 20 °C) was purchased from Sigma Aldrich. Anhydrous dimethyl sulphoxide (DMSO) with purity ≥ 99.9% and Sodium Phosphate for buffer solution was purchase from Sigma.

Ethanol and acetone with analytical pure grade of 99.0%, perchloric, oxalic, phosphoric acid, chromium trioxide and hydrargyrum chloride (Hg_2Cl_2) were purchased by Sigma Aldrich, Centricon® filters was supplied by Millipore.

Preparation of APA templates

APA electrochemical synthesis was performed as in [2, 7]. Aluminium foil with a thickness of 0.5 mm was cut to assume a rectangular shape. This template was used as anode in an electrolytic Teflon bath-cell to synthetized APA, with a second platinum counter electrode. Aluminium foil was degreased with ethanol and acetone and received an annealing heating in a stove for 4 hours at 420 °C. After this pre-treatment the sample's surface was treated with a mixture of ethanol/perchloric acid, ratio 1:4 v/v for 3 min in an electrochemical bath cooling setup (15 V, current density 15 A/dm^2) at 2 °C to remove dust impurity and the natural passivized oxidation effect on the surface. After this, aluminium rectangular sheet was connected to the positive electrode, while platinum foil to the negative electrode. The anodization time was chosen in function of the desiderated APA thickness. The electrochemical anodization was carried out for 9 h with vigorous stirrer to disperse the heat being developed in the electrolytic solution. In particular, the first anodization was performed for 1 h in a cooling system, able to maintain the temperature at approximately -4 °C, applying current voltage of 56 V, by using an aqueous oxalic acid electrolyte solution 1.0 M. After that, anode was rinsed with milliQ_water and then treated with a mixture of phosphoric acid and chromium trioxide, ratio of 1:4 (v/v) to remove the disordered aluminium oxide, growth during the first step. The second anodization was performed for the remaining 8 h. During this second anodization step the temperature was maintained at a 5 °C. The remaining aluminium was removed by Hg_2Cl_2 solution.

Fluorescein labelling of lysozyme

The labelling procedure was performed as described in [19]. 1.2 ml of CFSE with concentration 1.5 mg/ml in DMSO was added to 12 ml of lysozyme solution with concentration 1 mg/ml in phosphate buffer pH 7.4. The mixture was incubated for 90 min in a dark at room temperature with continuous and gentle agitation. Labelled protein was separated from free fluorescein by centrifugation with Centricon® filter for 30 min at 5000 rpm, and maintained at 4 °C repaired from light by aluminium foil cover. No precipitation appears after several days CFSE-labelled protein solution storage.

Protein confinement by APA template

Anodic porous alumina, before the drop cast, was maintain dried in a desiccator. Gilson pipette was employ to drop 1-2 µl of solution on the surface of anodic porous alumina. The sample distribution on the APA surface was performed by spin-coating apparatus Laurell WS-650 23b. Vacuum, obtain by rotary pump in the chamber assures perfect adherence of sample during rotation (10 min at 5000 RPM speed)

AFM measurements

Anodic porous alumina membrane was characterized by Bucker Edge AFM using a nano spider instrument in non-contact mode by Si conical tip with a typical curvature radius of 10 nm and an aspect ratio about 3:1. The average constant force in non-contact mode was 17 N/m.

Fluorescence intensity measurements

In order to characterize the fluorescence intensity from APA samples, CCD AxioCam camera (Carl

Zeiss) with Axio Vision software 3.0 was used to acquire the picture frame with working resolution of 1300 x 1030 pixels with various illumination levels by varying the exposure time (diaphragm aperture). Final picture was collected with 2560 x 1920 pixel (full HD) resolutions. Image j support software was used to normalize the fluorescence intensity. At beginning the native fluorescence of anodic porous alumina was detected as a blank in order to subtract it from the sample fluorescence, as anodic porous alumina itself shows a weak fluorescence, which can interfere with the fluorescence emission of the marker in use.

Raman spectroscopy

Raman spectroscopy was used to investigate the conformation of lysozyme on the anodic porous alumina as a single vibration group. For the calibration of the instrument (DILOR Z-24 Raman spectrometer) a Si band (520.7 cm^{-1}) was adopted; the range of the calibration was ~200-800 cm^{-1}.

Raman scattering was detected by CCD-camera cooled with a thermoelectrically cooling system (Peltier element) to -50 °C.

The incident laser light was focused on a region of 4 mm^2 using an incident wavelength at 532 nm objective 20X with NA 0.45, laser power 200 mW, grating 600 Lines/mm with spectral resolution 3.18 cm^{-1}. Smoothing of signal and base line subtraction was performed using Oregin software 9.2 (OriginLab Corporation, United States)

Results and Discussion

In figure 1, a fragment of APA sample is shown at 10X magnification, the surface appears compact and homogeneous. The black region in the figure defines the border of alumina. Some scratches on the surface are due to the metallurgic process used to extrude aluminium foils. This fragment was analyzed be means of AFM (Figure 2) Multiple channel of APA are quite ordered with some visible common pillar defects (crests on the pore's crowns) occurred during APA electrochemical synthesis. This microporous structure has been used to confine the labelled protein by spin-coating technique.

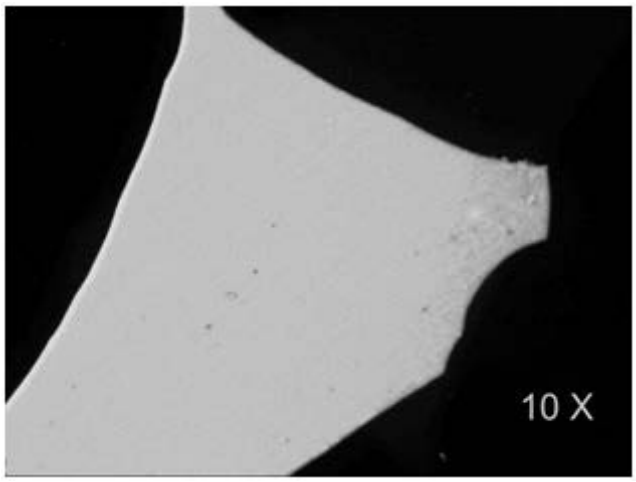

Figure 1: Anodic porous alumina fragment 10X magnification.

Two APA samples have been used: the first as reference to collect native APA fluorescence emission; the second as a template for labelled protein deposition.

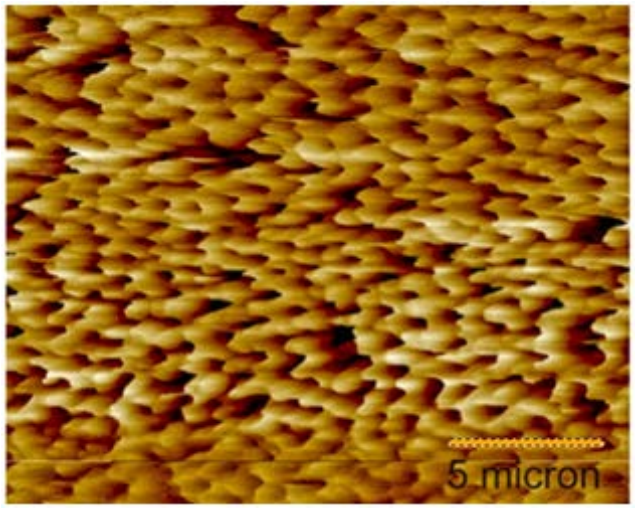

Figure 2: AFM image of APA with visible pore size on the microscale.

Automatic modality of CCD camera calculates the acquisition time, exposition, normalized fluorescence effect and gain from single frame. The image frames have been acquired at different value of diaphragm aperture. Different exposure time has been used for the data collection. Increasing of the acquisition time (time of exposure) from 0.2 to 1.2 ms with a diaphragm aperture results in increasing of intensity of fluorescence signal from the sample. The figure 3 is shown the APA green fluorescence with an exposure time of 1.3 ms and 20X microscope magnification. The small yellow square on the figure points to the area of fluorescence signal acquisition. In figure 3A is shown the native fluorescence of APA, which possibly derives from F+ centre in the structure of anodic porous alumina [20]. Some evident light spikes could appear due to corrupted defects (e.g. crest defects) on the surface

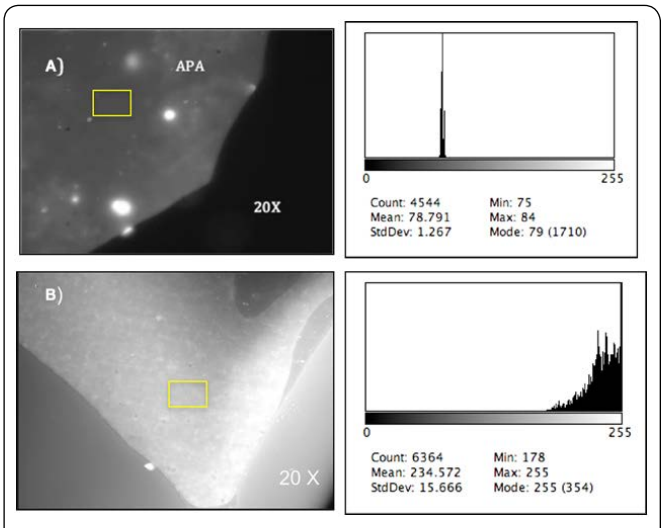

Figure 3: APA fluorescence images obtained by CCD camera with corresponding intensity histograms: **(A)** APA fluorescence at 20 X magnification **(B)** APA with CFSE-labeled lysozyme, incorporated in the APA pores at the same magnification.

of APA. In figure 3B the fluorescence signal of APA with CFSE-labelled lysozyme is shown. On the left side of the figure 3 the corresponding histogram of intensity is presented, confirming the drastic increasing of intensity of the sample with the labelled protein incorporated in comparison with clean APA surface.

A clear pore size distribution is evident from figure 4 with 40X magnification and corresponding intensity histogram. The signal was acquiring in the area of the pore (yellow square), confirming that the labelled protein molecules have been incorporated inside the pore. The same result was obtained from each APA pore, confirming that presence of labelled protein in the pores. The native APA fluorescence is relatively week in comparison with CFSE-labelled lysozyme and in case of quantitative analysis can be easily subtracted from the resulting intensity signal.

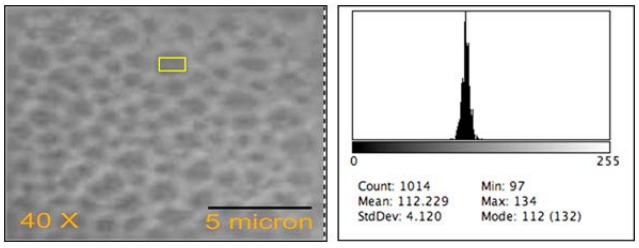

Figure 4: APA with CFSE-labeled lysozyme at 40X magnification and fluorescence intensity histogram.

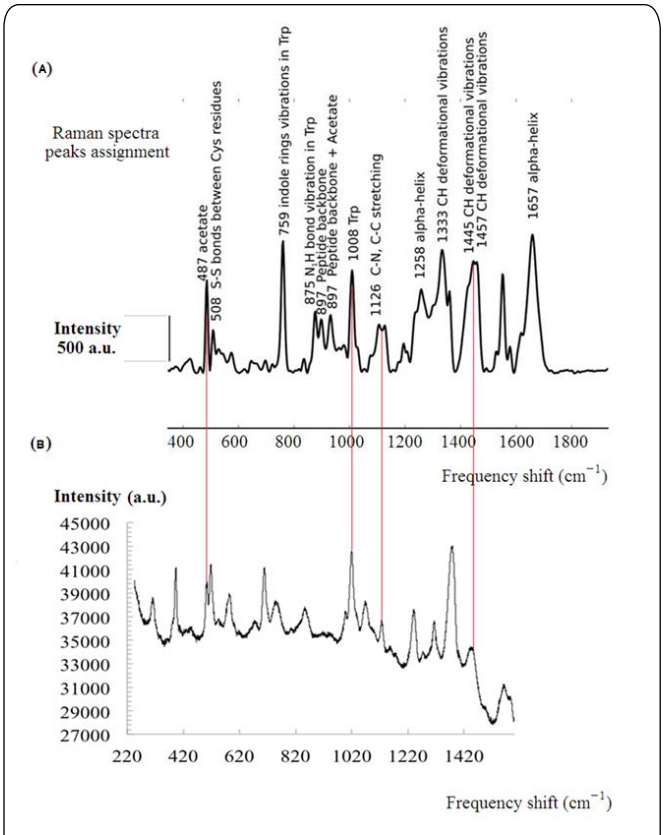

Figure 5: (A) Reference lysozyme Raman spectra with the peaks assignment obtained by our group in 2014 [21]; **(B)** Raman spectra of CFSE-labeled lysozyme, incorporated in the APA pores.

Further, we use Raman Spectroscopy to study the lysozyme conformation into the APA poses. Raman spectra of lysozyme is shown on the figure 5. We compare the Raman spectra of the lysozyme crystals, previously obtained [21, 22] with the CFSE-labelled lysozyme, confined in the APA pore. Indeed, the Raman spectra peak positions of CFSE-labelled lysozyme into the APA pore are similar to those obtained for non-labelled protein in crystals (see Figure 5). The lysozyme Raman spectra of 532 nm laser excitation with the peaks assignment [21] was taken as the reference one (Figure 5A), even if the frequency shift range of (400-1800 cm^{-1}) is slightly different in comparison with present experiment (200-1500 cm^{-1}) (Figure 5B). Moreover, position of peaks in Raman spectra of lysozyme are similar as we observed previously in lysozyme Raman spectra obtained by 632.8 laser nm excitation [22]. The peak corresponding to acetate (487 cm^{-1}) found in the obtained Raman spectra can result from the lysozyme buffer 50 mM Sodium Acetate pH 6.5.

Another difference of the present study from the previous one [22] is that spectral region was 500-1100 cm^{-1} instead of 200-1500 cm^{-1} in the present experiment. The obtained result confirms that lysozyme conformation into the APA pores generally corresponds to its native conformation found in crystals, and its confinement in the APA pore does not cause any damage to its secondary structure.

Conclusions

Nanostructuring of a porous alumina matrix for a bio-molecular microarray was at the onset of APA into Nanotechnology [23] with a classical protocol, in the present study APA have a more expanded volume during the growth of pores. Lysozyme labelled with fluorescence marker (CFSE) has been successfully confined in APA by spin-coating technique. While analysis of fluorescence intensity confirms protein incorporation into the pore, Raman spectroscopy data from labeled lysozyme in the APA template are in the agreement even with our most recent [24] results of classical and LB lysozyme crystals, confirming that no significant changes in the protein structure are caused by confinement. Therefore, the suggested approach allows optimal confinement of the protein molecules in APA channels and could find potential application in the protein microarray development both for diagnostic and for protein-protein interaction studies. Being the protein incorporation into the APA pores confirmed by high fluorescence intensity signal from the pore, the future developments will be focussing on the label-free protein-protein interaction study on APA array for an industrialized nanoapproach to cancer [25, 26].

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