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Use of Electrochemically Machined Porous Silicon to Trap Protein Molecule

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Abstract: Silicon surface chemistry is crucial to allow access to technologically interesting thin films not only for electronic industries but also for biological applications. Silicon wafers were etched electrochemically using ethanolic HF solution under different current densities (143 to 714 $mA.cm^{-2}$) while etching time was adjusted to obtain a constant charge of 4.5 $C.cm^{-2}$ to generate pores of different sizes (50 to 1500 nm). Atomic Force Microscopy (AFM) and Scanning Electron Microscopy (SEM) studies revealed the properties of porous layer such as pore distribution, diameter, geometric shape of the pores, which depend upon HF concentration, current density during electrochemical etching, temperature etc. Stability of the porous silicon wafer was achieved by thermal oxidation. In this study, attempted were made to determine whether porous silicon can be used to bind protein reversibly. Admittance and visualization of the protein (i.e. Bovine serum albumin) adsorption in pH gated admission experiments were determined by atomic force microscopy. It was observed that the 66 kDa BSA protein (~15 nm diameter) when positively charged (i.e. with citrate buffer, pH 4) would evenly coat the negatively charged surface of an oxidized porous silicon. The bound protein could be eluted under conditions that imparted a net negative charge on bound BSA (i.e., with phosphate buffered saline, pH 7). Thus, the electrochemically machined porous silicon can be used as molecular sieve to trap protein molecule, which could allow the *in vitro* study of protein-protein interactions.

Key words: Current density, etching, pore morphology, silicon wafer, thermal oxidation

INTRODUCTION

Silicon surface chemistry is of fundamental importance because of the ubiquitous role and technological importance in electronics industry due to the versatile semi-conducting properties of its crystalline phase and is crucial to allow access to technologically interesting thin films for fabrication of new electronic devices. Porous silicon was discovered in 1956 by Ulchir (Buda and Kohanoff, 1994) while studying the electro-polishing of silicon surfaces using hydrofluoric acid solutions. Further chemical modifications were explored leading Canham and co-workers in 1990 to the important discovery that highly porous silicon obtained by electrochemically etching a silicon wafer, would efficiently emit visible light at room temperature (Canham, 1990). Extensive experimental efforts have been carried out to characterize the relation between microstructure and luminescence in porous silicon (Cullis and Canham, 1991) as well as the stability and origin of the luminescence with a broad variety of techniques. Porous silicon is well-known, inexpensive, integrable with silicon technology, and very sensitive to the environment due to its large surface versus volume ratio (Canham, 1997; Baratto *et al*., 2002). Such promising experimental approaches may represent the beginning of a new technological era in the application of silicon based materials.

Size exclusion chromatography is a common technique used to resolve mixtures of proteins and other macromolecules. The basic principle is based on the molecular size dependent distribution of analytes, where the mobile phase carries the analytes through a given type of stationary porous matrix in a chromatographic column. If the analyte is large, it is excluded out from the stationary phase and hence comes out of the column faster. On the other hand, if the analyte is sufficiently small, it gets access within the pores of the stationary phase and experiences a larger path length, and thus comes out of the column later. Hence, these two limits make possible the separation of an analyte mixture with a narrow molecular size range in an appropriate porous stationary phase.

Porous silicon is a nanocrystalline matrix that is formed by electrochemical oxidation of silicon in aqueous ethanolic hydrofluoric acid (HF) solution, which consists of nano or microcrystalline domains displaying a distribution of well defined pore morphologies (Collins *et al*., 2002). The properties of the porous layer such as pore size, porosity, diameter, geometric shape and direction of the pores depend upon several factors like type and amount of dopant on the surface, concentration of HF, current density during the electrochemical etching, temperature *etc* (Searson, 1994; Collins *et al*., 2002). The porosity of the porous silicon layer i.e., the proportion of void with respect to bulk silicon, as well as the average pore diameter increases linearly with the applied current densities (Buda *et al*., 1992; Buda and Kohanoff, 1994).

The pore dimensions are commensurate with those of protein molecules. Nanocrystalline porous silicon films

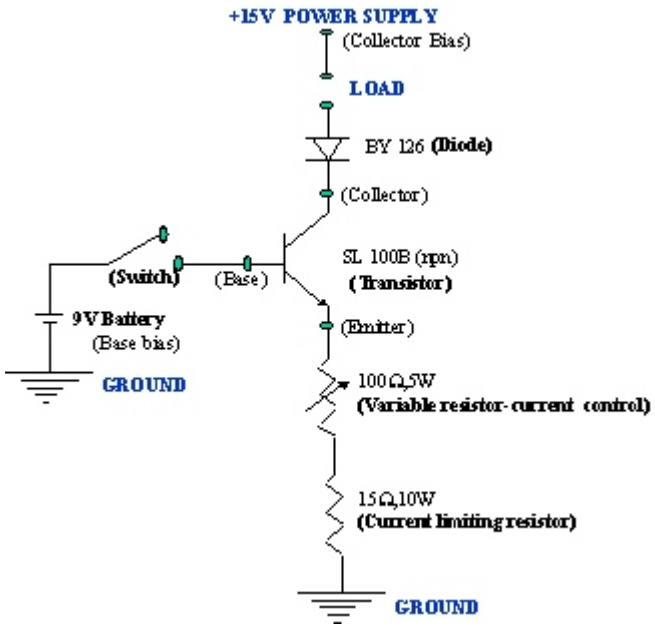


Fig. 1: Circuit employed to provide constant current for electrochemical etching of silicon wafer

can be used as chromatography matrices (Higanaka *et al*., 1990; Letant and Sailor, 2001; Strange *et al*., 2003; Chen *et al*., 2004; Lin *et al*., 2007; Anglin *et al*., 2008). Together they present the possibility of coupling the detection and separation processes as simultaneous events (Lin *et al*., 2007). Porous silicon has been employed as a large surface area matrix for the immobilization of a variety of molecules (Lin *et al*., 2007; Schwartz *et al*., 2007; Jang *et al*., 2008; Park *et al*., 2009) including enzymes (Drott *et al*., 1998, 1999; Bengtsson *et al*., 2002; Letant *et al*., 2004; Orosco *et al*., 2009) and antibodies (Laurell *et al*., 1999). Internal-surface Reversed-phase Silica (ISRP) support was used to determination of hydrophilic or hydrophobic drugs in serum or plasma (Higanaka *et al*., 1990; Letant and Sailor, 1999; Jang *et al*., 2008) and drug delivery (Chen *et al*., 2004; Anglin *et al*., 2008). Microfabricated devices operated in a flow-through mode over flat surface design containing arrays of nucleic acid hybridization sites, known as genosensors, are being developed for a variety of uses in genomic analysis (Beattie *et al*., 1995). In addition, porous silicon has been identified as a potential optoelectronic material (Gao *et al*., 2000; Youssef, 2001; Baratto *et al*., 2002; Alwan, 2007) and used as pressure sensor (Sujatha and Bhattacharya, 2009). It is thus of primary interest to determine the possible configuration in which porous silicon flims can be used to trap protein molecules according to their three dimensional structure and size, which would have immense practical utility in the study of biology.

MATERIALS AND METHODS

The study was conducted in the Department of Biological Sciences and Department of Condensed matter Physics when I was perusing my visiting student research programme during 2002 at Tata Institute of Fundamental Research, Mumbai. Further, I have taken the help of Central Sophisticated Analytical Instrumentation Facility at TIFR for taking Atomic force microscopic and Scanning electron microscopic images to characterize the freshly etched silicon wafer for the pH gated admission experiment.

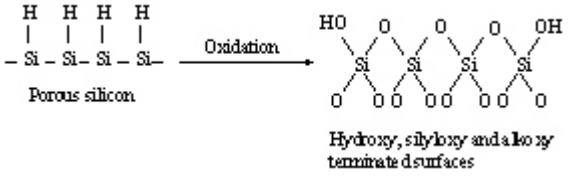
Circuit used for Etching: The basic need of the circuit (Fig. 1) was to provide a continuous constant current supply for etching the silicon wafer at different current densities. In this circuit, the input power supply is 15 V and source voltage was positive referred to as collector bias. The instrument used for etching of silicon wafer was connected in series with the circuit. The diode (BY-126) used in this circuit can be thought of as a switch, which is controlled by the polarity of the source voltage. The diode conducts the current only from (p) to (n) and conduction takes place only when the source voltage is positive. In addition, a transistor made of up semi-conducting materials (SL-100B) was used for current amplification, which was initiated in base current rather than emitter current. A 9V battery is connected to the base of the transistor was referred to as base bias. Besides these; a variable resistor current control (100W, 5W) was used in the circuit by which the current supply required for etching was manipulated as per the requirement and a current limiting resistor (15 Ohm, 10 w) was used to maintain the overall current supply connected to the ground.

Electrochemical preparation of porous layer: Heavily doped p-type silicon wafers of orientation (100) with resistivities within a range of 0.6-1.0 m Ohm cm were

used to fabricate porous silicon. Prior to etching, silicon wafers were rinsed and washed with trichloroethylene to remove surface impurities. In addition, the wafers were subjected to ultrasonic cleaning for 2 min followed by boiling for 2 min in trichloroethylene. The silicon wafers were then dried under a stream of nitrogen and dipped in concentrated HF solution to remove any remaining impurities. The silicon films were washed thoroughly with absolute ethyl alcohol and dried under a stream of nitrogen. A silicon wafer of an exposed area of about 0.7 cm^2 was mounted in a teflon etching shell (Fig. 2). A platinum mesh served as the counter electrode used to provide a homogenous electric field. Etching was performed in ethanolic HF solution (HF : EtOH = 3:1, v/v) prepared with 48% HF in water. Galvanometric anodization was performed with current densities ranging from 143 to 714 $mA.cm^{-2}$ while etching time was adjusted to obtain a constant charge of about 4.5 $C.cm^{-2}$. After etching, the films were rinsed thoroughly with absolute ethanol and dried under a stream of nitrogen.

Characterization of porous silicon films: To characterize the porous silicon films, atomic force microscopy (AFM) images were obtained using a Nanoscope–IIIa multimode scanning probe microscope operating in tapping mode, measuring the surface topography of the porous silicon by recording the feedback output and the cantilever deflection, which can achieve resolution upto 10 pico meter. In addition, Scanning Electron Microscopy (SEM) image of cross section of the freshly etched silicon film was also obtained with Cambridge 360 electron microscope using an accelerating voltage of 20 KeV to determine the pore dimension within the porous layer.

Oxidation of porous silicon: The freshly etched porous silicon was thoroughly rinsed with absolute ethanol and dried under a stream of nitrogen. Thermally oxidized porous silicon samples were obtained by heat treatment in a furnace tube using the following parameters: initial ramp temperature $5^oC.min^{-1}$ to 400ºC, holding time - 1 h and passive cooling to room temperature.



pH Gated admission experiment: The oxidized porous silicon films were initially washed with a 10% (v/v) solution of ethanol in deionized water followed by the buffer solution (*i.e.* citrate buffer, pH 4.0). Then, 100 µl of the BSA (i.e., Bovine Serum Albumin) protein sample consisting of 1 $mg.ml^{-1}$ protein (15mM) in citrate buffer (pH 4.0) was gently introduced over the surface of the porous silicon wafer. Non-specifically adhered protein

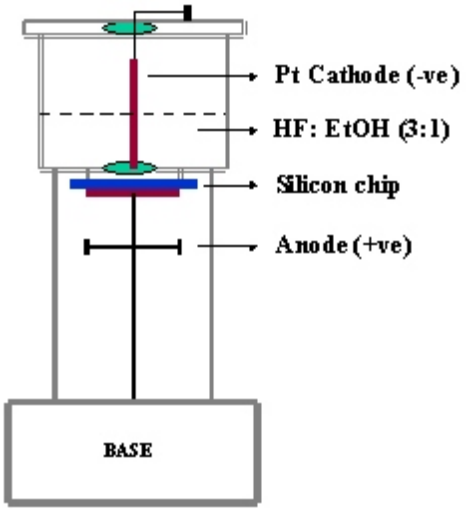


Fig. 2: Diagrammatic representation of the etching cell used for electrochemical etching of silicon wafer.

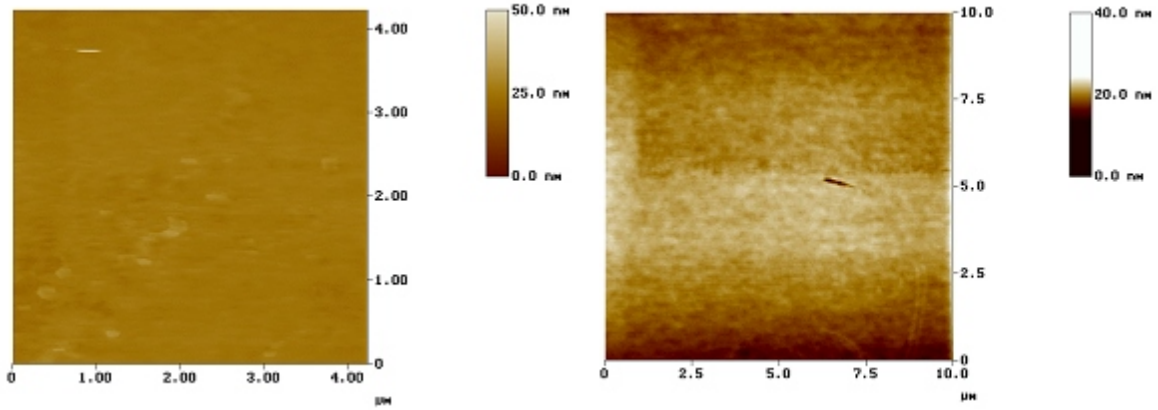
Table 1: Electrochemical etching of silicon wafer at different current densities (143 to 714 $mA.cm^{-2}$) generating pores of 50 nm to 2000 nm pore diameter.

Current Density ($mA.cm^{-2}$)	Pore Diameter(nm)
143	5 – 50
285	50 -100
357	100 – 150
428	200 – 400
500	500 – 800
571	800 – 1000
642	1000 – 1500
714	Electropolishing

was removed by a further washing with citrate buffer (pH 4.0). Impregnation of the BSA molecules into the porous silicon film was examined by atomic force microscopy and fluorescence spectroscopy. The porous silicon used in this experiment had pores size of 1000 nm that was large enough to accommodate the BSA protein (15 nm). Phosphate buffered saline (pH 7.2) was then introduced on the surface of the porous silicon to elute out the BSA trapped on the oxidized silicon surface. Further, the protein bound to the surface of the silicon wafer was estimated using front-face fluorescence spectroscopy.

RESULTS AND DISCUSSION

Pore Morphology: When current flows in the electrochemical cell, the dissociation reaction localizes on a particular side of a silicon surface, thus initiating the etching of an array of pores in the silicon wafer. The pore morphology was analyzed under conditions of varying current densities. At low current density, a highly branched, randomly directed and highly inter-connected meshwork of pores was obtained. However, increasing in current density orders the small pores to exhibit cylindrical shapes giving rise to larger pore diameter


50.0 nm
25.0 nm
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2.00
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0
1.00
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40.0 nm
20.0 nm
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2.5
5.0
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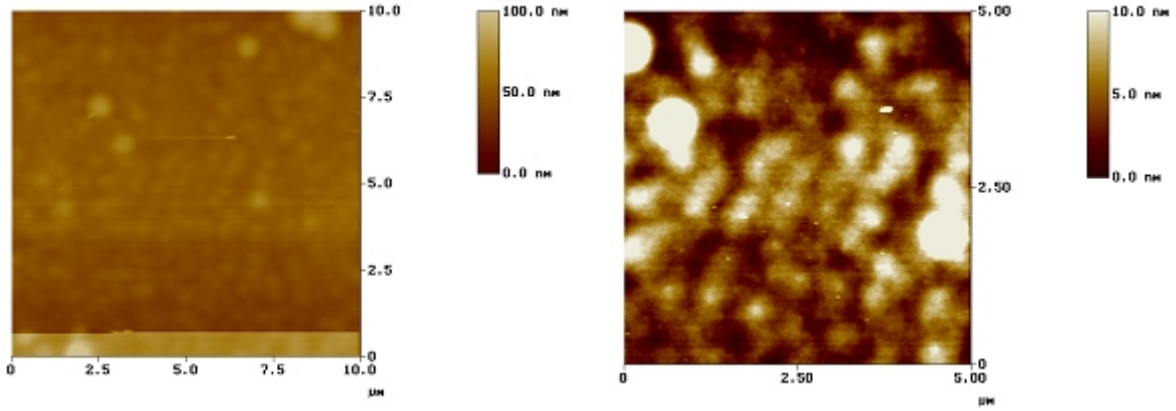
μm

(A) (B)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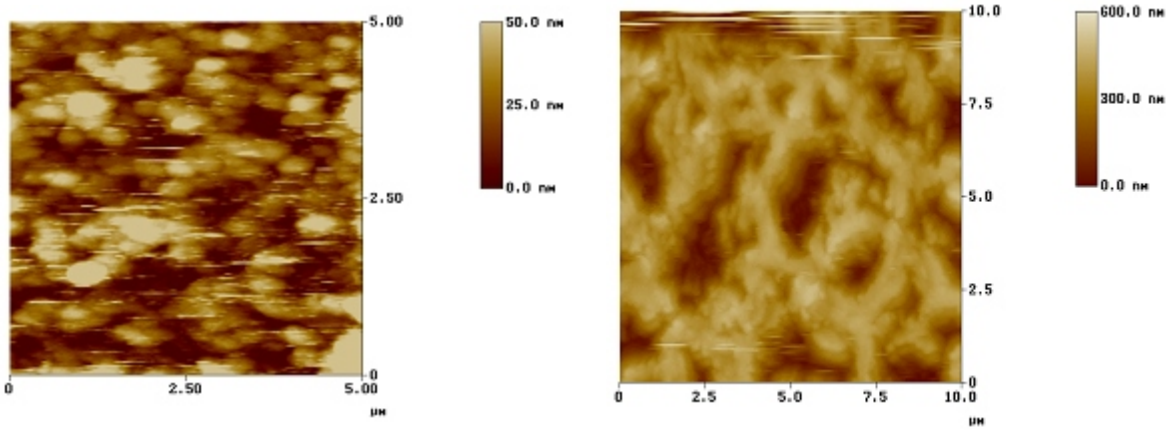
(C) (D)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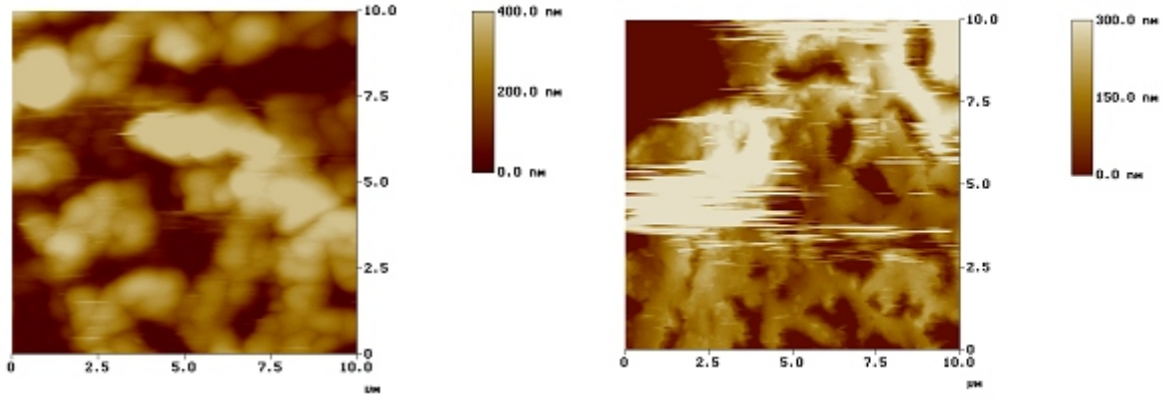
(E) (F)



(G) (H)

Fig. 3: AFM images of porous silicon layers etched at different current densities: (A) 143 mA.cm^{-2} (B) 285 mA.cm^{-2} (C) 357 mA.cm^{-2} (D) 428 mA.cm^{-2} (E) 500 mA.cm^{-2} (F) 571 mA.cm^{-2} (G) 642 mA.cm^{-2} (H) mA.cm^{-2}. All samples were etched to a constant charge of 4.5 C.cm^{-2}.

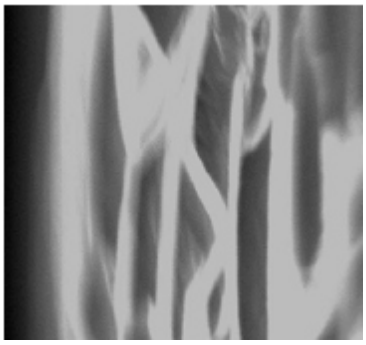


Fig. 4: Cross-sectional SEM image (1 x 1 μm^2) of porous silicon etched at 571 mA.cm^{-2} for 8 sec.

(Buda and Kohanoff, 1994; Beattie *et al.*, 1995; Canham, 1997; Collins *et al.*, 2002). The study revealed that the current densities used in this experiment ranged from 143 to 714 mA.cm^{-2} supplemented a good gradation of pore diameters varied from 50 to 1500 nm (Table 1). Eventually, the detachment of the porous layer occurs at current density higher than 700 mA.cm^{-2} referred to as 'electro-polishing' (Collins *et al.*, 2002) (Fig. 3, A-H). Besides these, the cross sectional image of a freshly etched porous silicon showing the characteristics cylindrical pore morphology describes the pores as a bundle of cylinders (Fig. 4). It is difficult to obtain reliable information about the pore size from the samples with pores smaller than 5 nm because in this size regime the apparent pore size strongly depends on shape of the SEM probe and the threshold height chosen. Imaging the larger macro-pores (> 800 nm) can result in unstable scans because the tip loses contact with the surface over a relatively long distance. In particular, the large macro-pores have blackish appearance due to the predominant interaction of the side of tip and with the pore wall.

The study suggested that the pore morphology and dimension depends upon the etching condition, applied voltage and HF concentration (Canham, 1990, 1997; Collins *et al.*, 2002). The pore morphology is also determined by a combination of electric current and a characteristic non-linearity parameter, which depends on specific properties of the substrate like dopant level and temperature (Canham, 1997; Searson, 1994; Collins *et al.*, 2002). The doping of the silicon wafer modifies its resistivity and consequently the local electrical field inside the anode (Canham, 1990; Buda *et al.*, 1992; Youssef, 2001).

Oxidation of porous silicon: The surface of the freshly etched porous silicon film was predominantly hydride-terminated and unstable in aqueous solution, readily suffers oxidative and hydrolytic corrosion. Therefore, to obtain a stable porous layer surface in an aqueous buffer, the porous layer was subjected to oxidation prior to use. Thermal oxidation converts the hydride terminated porous silicon surface into a stabilized form by the incorporation of oxygen into the silicon crystal lattice and gives termination with silyloxy (Si-O-Si), hydroxy (Si-OH), alkoxy (Si-OR) groups or a combination of these three functionalities (Robins *et al.*, 1999; Letant and Sailor, 1999). Again, hydroxy (Si-OH) terminated surfaces can then undergo condensation reaction with alkoxy- or chlorosilanes to produce a new (Si-O-Si) linkage covering the porous surface with a layer of oxide (SiO_2) that was negatively charged even at moderately low pH values (Canham, 1990, 1997; Searson, 1994; Collins *et al.*, 2002).

pH Gated admission experiment: In order to adsorb the BSA (i.e., Bovine serum albumin) protein on the negatively charged surfaces of oxidized porous silicon

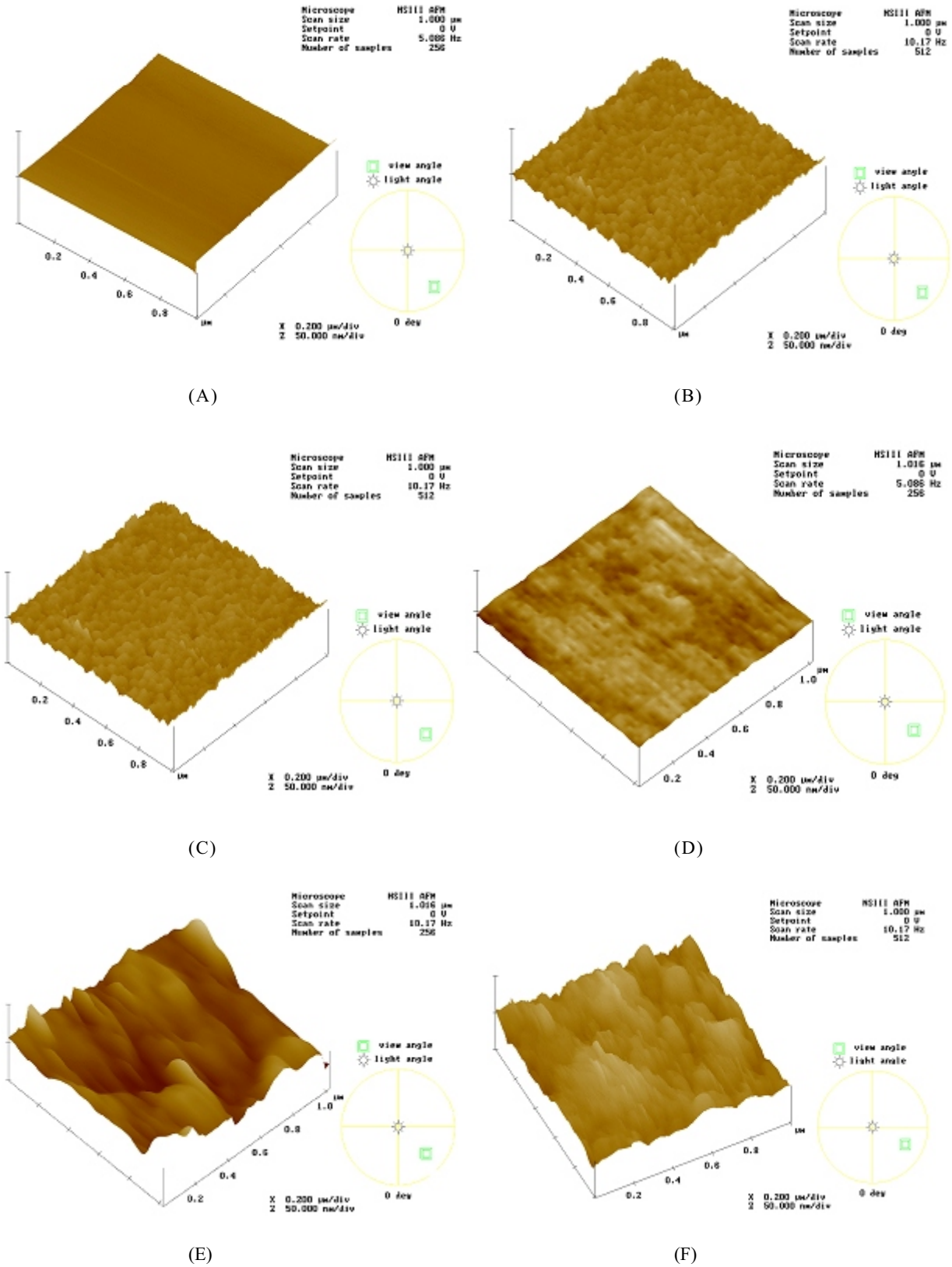


Fig. 5: AFM images demonstrating the pH gated adsorption of BSA on Porous Silicon by monitoring surface topography. (A) silicon wafer without etching; (B) oxidized porous silicon; (C) oxidized porous silicon washed with 10% EtOH; (D) oxidized porous silicon washed with citrate buffer of pH 4; (E) oxidized porous silicon showing trapped BSA protein in pH 4 buffer solution (F) after washing with pH 4 buffer. The silicon wafer was etched at 642 $mA.cm^{-2}$ for 7 sec

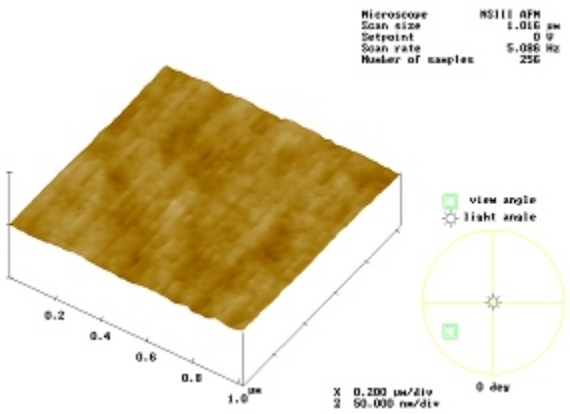


Fig. 5: (continued). AFM images of (G) after elution of BSA with phosphate buffered saline, pH 7.2

films, the protein must bear a net positive charge. The isoelectric point of BSA is approximately 5. BSA will thus display net positive charge at solution pH value below 5 *i.e.* with citrate buffer (pH 4.0). By the same token, it will bear a net negative charge with phosphate buffered saline (pH 7.2) due to the pK_a values of the constituent amino acid residues. These considerations were used to selectively adsorb positively charged BSA on the negatively charged oxidized porous silicon surface at pH 4 and to elute it off under conditions of pH 7.2. Thus, the electrostatic interaction between the SiO_2 covered pore surface and the protein can be varied allowing the adsorption and release of the protein to be experimentally controlled (Canham, 1990; Collins *et al*., 2002). As because BSA contains three tryptophan residues that could serve as intrinsic fluorophores, the protein bound to the surface of the silicon wafer was estimated using front-face fluorescence spectroscopy to determine the emission of tryptophan fluorescence (Excitation: 280 nm, Emission: 340 nm) proved successful.

Further, attempts were made to read-out of adsorbed BSA protein in terms of three-dimensional representation of a (1 x 1) μm^2 area of porous silicon wafer subjected to various treatments using atomic force microscopic studies. The AFM study suggested that the silicon wafer prior to etching showed relatively flat and featureless surface (Fig. 5A) while blurring of the surface with hills and valleys corresponding to the pore distribution obtained by etching the silicon at a current density of 642 $mA.cm^{-2}$ (Fig. 5B). This surface was washed with 10% ethanol and visualized using AFM (Fig. 5C), which shows no difference. The image obtained after washing silicon wafer with citrate buffer (pH 4.0) looks quite different (Fig. 5D), because citrate buffer is not volatile like ethanol and thus sits trapped on the ridged surface of the silicon wafer. When BSA was introduced, the protein seems to cover the entire landscape of the porous silicon surface like a blanket (Fig. 5E). Washing away the non-specifically adsorbed protein using citrate buffer (pH 4.0) results in a somewhat sharper landscape (Fig. 5F) still showing considerable protein covering the porous silicon. Spectacularly, the subsequent washing of silicon wafer with phosphate buffered saline (pH 7.2) completely removes the adsorbed BSA protein (Fig. 5G) resulting in a surface topography similar to that observed in Fig. 5D prior to the admission of the protein. The study revealed that the electrochemically machined porous silicon can be used as the stationary phase as in chromatographic separation (Higanaka *et al*., 1990; Lin *et al*., 2007; Gritti and Guiochon, 2009).

CONCLUSION

The above experiments convincingly demonstrate that protein can be adsorbed onto the surface of porous silicon in a controlled manner, where the adsorption and elution from the negatively charged pore surface can be determined by the net charge on the biomolecule. This property in itself can be used to study biological interactions of macromolecules with one another. The extremely high surface area within a small volume provided by porous silicon results in very high effective concentrations of protein adsorbed on these surfaces. This could allow the *in vitro* study of protein-protein interactions of physiologically effective protein concentrations, where proteins are often sequestered within two-dimensional membrane micro-domains thereby greatly increasing their effective concentrations. Furthermore, the net negative charge on the surface of oxidized porous silicon can conceivably be neutralized by treatment with positively charged small ligands like poly-lysine, which could then prevent charge-based surface adsorption of proteins on the porous silicon and may be used as a macromolecular sieve. This study further supplemented that a gradient of electrochemically machined porous silicon can be used not only to trap protein molecules, but also separation of proteins as simultaneous events.

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