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Characterization of cluster-assembled nanostructured titanium oxide coatings as substrates for protein arrays

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ABSTRACT

Protein microarray technologies are rapidly expanding to fulfill current needs of proteome discovery for disease management. Nanostructured materials have been shown to present interesting features when used in biological settings: nanostructured titanium oxide film (ns-TiOx), synthesized by supersonic cluster beam deposition (SCBD), has recently emerged as a biocompatible substrate in different biological assays. The ns-TiOx surface is characterized by a morphology at the nanoscale that can be tuned to modulate specific biomolecule–material interactions. Here we present a systematic characterization of ns-TiOx coatings as protein binding surfaces, comparing their performances with those of most common commercial substrates in protein and antibody microarray assays. Through a robust statistical evaluation of repeatability in terms of coefficient of variation (CV) analysis, we demonstrate that ns-TiOx can be used as reliable substrate for biochips in analytical protein microarray application.

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The complexity of the human proteome has encouraged the development of sophisticated multiplexed technologies for more appropriate methods of analysis. As a consequence, the trend of detection technologies has moved from low-throughput analysis (e.g., enzyme-linked immunosorbent assay [ELISA],[1] Western blots, mass spec) to "high-content/high-throughput" approaches relying either on planar- or "bead"-based solutions [1–4]. In this context, the development of novel miniaturized devices, such as biochips and microarrays, can offer a valid approach to interrogate the proteome by multiplexing the information at a reasonable cost.

The successful development of such devices relies on the optimization of the complex interactions that occur between "proteins" and the "surface" that provides the means for immobilization. The modalities of protein adsorption are quite different, according to the variety of the substrates (e.g., two-dimensional, three-dimensional, covalent binding, electrostatic, diffusion), and deeply influence the performance of the assay. Moreover, substrate fluorescence background, assay reproducibility, and affinity toward specific targets (which influence limit of detection and linearity) are distinctive features of protein microarray assays, which require precise selection of optimal conditions [5–7]. In this regard, the availability of novel surfaces for protein immobilization represents an opportunity for the setting up of microarray-based devices for optimized protein profiling [8–12].

The role of the surface nanostructure is the object of increasing interest so as to understand how this affects the surface–protein interaction in view of the nanoscale engineering of substrates to obtain better performing protein microarrays. Despite the wide effort in addressing the mechanism and role of surface topography in protein–biomaterial interaction, this topic is still a matter of controversial debate, probably due to the complexity of models that are evaluated in different studies [13–15]. We recently characterized cluster-assembled nanostructured titanium oxide (ns-TiOx) thin films produced by supersonic cluster beam deposition (SCBD) [16,17] as a biocompatible [18] and optimal substrate for cell microarray-based assays [19]. Furthermore, we also showed that cluster-assembled ns-TiOx can efficiently bind streptavidin by adsorption [20], underlying a putative role of surface nanoscale and chemistry, thereby suggesting that cluster-assembled ns-TiOx surfaces can be of interest for protein microarray applications.

Here we present a systematic evaluation of cluster-assembled ns-TiOx slides on protein microarray assays in comparison with commercial slides. We characterized the spot morphology regularity, and we compared the protein adsorption efficiency, intraslide

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[1] *Abbreviations used:* ELISA, enzyme-linked immunosorbent assay; ns-TiOx, nanostructured titanium oxide; SCBD, supersonic cluster beam deposition; AFM, atomic force microscopy; PBS, phosphate-buffered saline; NC, nitrocellulose; PMT, photomultiplier tube; IgG, immunoglobulin G; S/N, signal-to-noise; CV, coefficient of variation; M-CV, mean intraslide CV; W-CV, overall intraslide repeatability; A-CV, mean interslide repeatability; B-CV, general estimate of interslide repeatability.

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repeatability, and interslide repeatability to assess the use of ns-TiOx slides as a reliable protein microarray substrate.

Materials and methods

ns-TiOx slide deposition by SCBD

ns-TiOx coatings (50 nm thickness) were prepared on standard glass slides (Nexterion Glass D, clean room cleaned, Schott, Mainz, Germany) by depositing under high vacuum a supersonic seeded beam of TiOx clusters produced by a pulsed microplasma cluster source, as described in detail in Refs. [16,17]. After ns-TiOx deposition, slides were immediately annealed at 250 °C overnight in a clean oven, as described previously [20]. Coated slides were then packaged in vacuum and stored at room temperature in the dark until use.

The surface morphology of cluster-assembled ns-TiOx coatings was characterized by atomic force microscopy (AFM), employing a Digital Instruments Nanoscope multimode IV atomic force microscope in tapping mode.

Protein and antibody array assays

For protein arrays, streptavidin (Cy3 conjugated, Amersham–GE Healthcare, UK) was reconstituted and stored in phosphate-buffered saline (PBS, pH 7.4, Lonza Group) at 2 mg/ml at −20 °C and then diluted in spotting buffer with PBS and 5% (w/v) glycerol (BDH Biosciences) to a final concentration of 0.5–500 μg/ml and spotted using a BioDot AD1500 equipped with Axsys software.

Commercial epoxy-coated slides (SuperEpoxy slides, Telechem International, USA) and nitrocellulose (NC) slides (FAST slides, Whatman, GE Healthcare, UK) were treated according to manufacturer specifications. Slides were spotted at 50% humidity at 40 nl/spot with 2 mm pitch and 20 replicates per concentration.

After 3 h of incubation in 75% humidity, ns-TiOx slides were blocked with 5% (w/v) milk in 0.5% PBST (0.5% PBS/Tween 20) (BDH Biosciences) for 1 h. After blocking, slides were washed twice with 0.5% PBST, then with PBS, and finally with deionized H_2O to remove salts. Slides were then analyzed with a GenePix 4000B scanner at a 532 nm wavelength with 33% laser power and an appropriate photomultiplier tube (PMT) gain for each substrate (epoxy: PMT 750; ns-TiOx: PMT 750; NC: PMT 300) at 10 μm/pixel of resolution.

For antibody array experiments, mouse immunoglobulin G (IgG) antibodies (Jackson ImmunoResearch, USA) were diluted in two different spotting buffers containing PBS/5% (w/v) glycerol or PBS/1.5 M betaine (Sigma–Aldrich, USA) at final concentrations of 0.5–1000 μg/ml.

Slides were spotted at 75% humidity at 40 nl/spot with 2 mm pitch and 20 replicates per concentration. Epoxy-coated and NC slides were processed for incubation, blocking, and detection following manufacturer instructions; ns-TiOx slides were incubated for 5 h in 75% humidity and then blocked for 1 h in 0.5% PBST in 5% milk. At the end of blocking, slides were washed twice with 0.5% PBST and then incubated overnight with 1 μg/ml goat anti-mouse IgG (Cy3 conjugated, Jackson ImmunoResearch) in 1% (w/v) milk in 0.5% PBST.

Slides were then washed twice with 0.5% PBST, then with PBS, and finally with deionized H_2O. Slides were then analyzed with a GenePix 4000B scanner at a 532 nm wavelength with 33% laser power and appropriate PMT gain for each substrate (epoxy: PMT 300; ns-TiOx: PMT 300; NC: PMT 150) at 10 μm/pixel of resolution.

Data were exported to Microsoft Excel software for processing. The fluorescence at every spot was evaluated as the median of all measured pixels so as to reduce the influence of possible outlayers. The estimate of the fluorescence given at every concentration resulted from the average of 18 to 20 replicates (i.e., 18–20 different spots in the same experimental condition) according to the microarray assay. Signal-to-noise (S/N) ratio was calculated as the ratio between the mean spot intensity and the standard deviation of background intensity.

Statistical analysis of microarray data

According to recent work on quality control of microarray experiments [21,22], we evaluated the repeatability of both microarray assays by means of the coefficient of variation (CV) because it expresses the "dispersal of data around the average" (the standard deviation) weighted by the average value (so as to make comparable the amounts of dispersal at low and high concentrations). CV values were first calculated, for each slide, by taking into consideration the (median) fluorescence values measured at all spots separately for each given concentration.

Intraslide repeatability

By gathering the results of each set of four to six experiments (2 slides/experiment) related to the different assays (mouse IgG and streptavidin–Cy3 arrays) and conditions (spotting buffers), it is possible to average among slides the "internal" CVs at every concentration, obtaining a series of mean intraslide CV values (hereafter M-CV) ± 1.96 standard error (SE). The sequence of these M-CVs allows us to draw the pattern of variation of intraslide repeatability according to the different spotted protein concentrations. Moreover, we can obtain a single parameter representing the overall intraslide repeatability (hereafter W-CV, where W represents "within") by averaging all of the M-CV values, indifferently from the concentration given.

Interslide repeatability

If we take into consideration the average median fluorescence per concentration in any slide (same experimental conditions) and then we calculate the CV of those values among slides, we obtain a reliable estimate of interslide repeatability (A-CV, a single value at any concentration). By averaging the values referring to all A-CV values at different concentrations, we can also obtain a

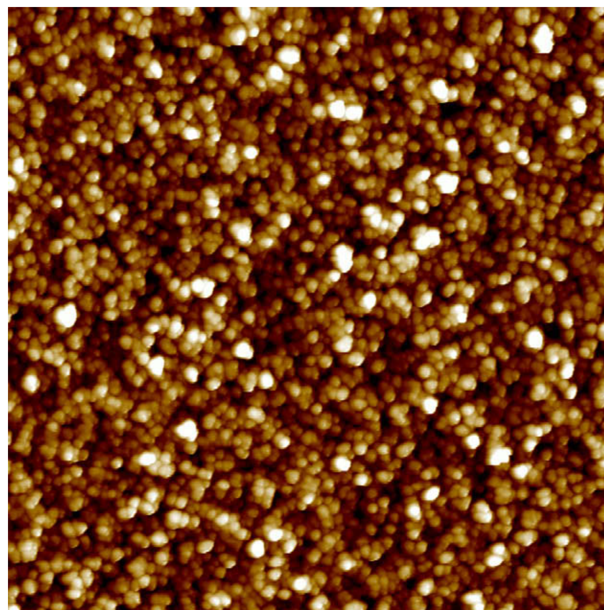

Fig. 1. AFM surface topography of a typical 50-nm-thick ns-TiOx coating deposited on glass coverslip (500 × 500 nm^2, vertical scale 15 nm).

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