Nanomaterials Can Influence Living Biological Systems with Nanometer Sensitivity

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ABSTRACT

We show the different response of human neuroblastoma cells line (SH-SY5Y) to gold surfaces with different levels of nanoroughness, finding out that neurons are capable to sense and actively respond to these nanotopography features. Particularly, focal adhesion complexes cannot assemble onto nanostructured surfaces, leading to a marked decrease in cell adhesion. We demonstrated that nanoscale features induce cell death by necrosis, with a trend directly related to the increasing roughness. By seeding SH-SY5Y cells onto micropatterned flat/nanorough gold surfaces, we realized substrates with cytophilic or cytophobic behavior, inducing a clear self-alignment of neurons only in the desired regions of the patterns. These substrates were also investigated to explore their use as novel materials which can prevent bacterial colonization. We show that surface nanotopography can lead to dramatic changes in adherent bacteria, such as variations in their morphology as well as genomic and proteomic profiles.

Keywords: nanotopography, neurons, bacteria, adhesion, biomaterials

1 INTRODUCTION

Biomaterials are regularly used in medical applications, such as orthopedic implant, tissue engineering, drug delivery systems, and medical imaging [1]. For this reason, numerous organic and/or inorganic materials have been specifically exploited to fabricate devices which may promote tissue growth, delivery of drugs, and avoid bacterial persistance. In such a frame, it has long been recognized that the intrinsic material properties may strongly affect biological outcomes [2]. Initially, cell-material interactions were tackled only from a chemical point of view, since environmental sensing by cells involves specific binding between cellular receptors and extracellular matrix (ECM) ligands. However, increasing evidence is recently showing that the biological response is also affected by the physical properties of the material [2]. From this perspective, cellular response to external stimuli includes a wide range of physical cues that are generated at, or act on, the interface between cells and the surrounding environment, and thus goes far beyond the bare ability of the cell to chemically sense specific ECM ligands. However, cell-substrate interactions are typically governed by complex mechanism occurring at the nanoscale, which are generally referred to as nano-biointeractions. In addition to eukariotic cells related responses, the physical properties of the materials have been also recognized to play a crucial role in bacteria-surface interactions. Such issue is of high importance since most of the implantable medical devices are prone to infection caused by bacteria that first adhere onto surfaces and then start to colonize and form hazardous biofilms [3].

In this work we show how nanotopography may modulate different biological systems, namely neurons (SH-SY5Y) and bacteria (*Escherichia coli*) cells, tailoring specific biological responses. In particular, we demonstrated that nanostructured gold surfaces can alter neurons behavior, with a direct dependence on surface nanoroughness [4]. Interestingly, such nanotopographies are able to trigger neuron adhesion/viability, with a surprising sensitivity of cells to nanometer-scale changes [4]. On the other hand, we investigated the response of *E. coli* cells onto flat and nanostructured gold surfaces, detecting significant differences in the bacteria growing onto the nanorough surfaces, mainly in terms of morphological aspects, along with the genomic and proteomic profiles [5].

We used a multidisciplinary approach to investigate such cells-biomaterials interactions, ranging from nanofabrication techniques (combination of optical lithography and wet chemistry) to molecular biology (RT-qPCR), biochemistry (2D-DIGE), and imaging (confocal, AFM, SEM).

2 NANOFABRICATION AND SUBSTRATES CHARACTERIZATION

To obtain a good control of surface topography, we exploited a wet chemistry approach, namely the redox-potential based metal coating, Spontaneous Galvanic Displacement Reaction (SGDR), that can allow a precise control over the surface morphology. SGDR techniques have, in fact, attracted great interest because of their simplicity of operation, cost effectiveness, high throughput, and lack of elaborate equipment, which make them good candidates for several commercial electroless plating processes. SGDR enabled us to reliably fabricate surface nanoscale features with accurate and controlled levels of surface nanoroughness, either uniformly extended over wide regions or confined only in some desired areas of the substrates (patterned samples). Briefly, by tuning the

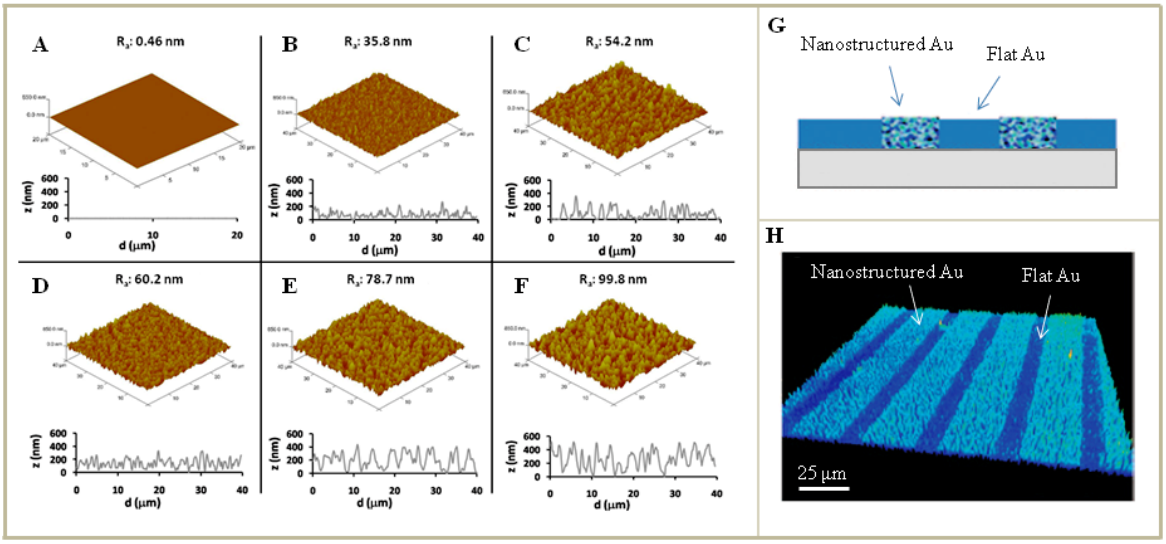

Figure 1: Substrates characterization. (Left) AFM analyses of flat and nanorough Au surfaces: an increase in R_a values is evident from picture A to F. (Right) Representative scheme (G) and holographic microscopy characterization (H) of 20 μm periodic stripes of alternated flat and nanostructured Au regions.

thickness of the initial sacrificial metal film (a thermal predeposited Ag film onto aminopropyltriethoxysilane modified glass slide), and then adding the exchange ions (typically $HAuCl_4$ 10^{-3} M for each sample), we were able to obtain Au surfaces with increasing values of surface roughness, with nanometer control and appreciable uniformity. Fig. 1 depicts the Atomic Force Microscope (AFM) analyses of the nanostructured surfaces employed for the cell growth. It is interesting to note the precise control of the surface nanoroughness, with mean roughness values (R_a) increasing from *c.a.* 36 nm of the first nanostructured sample (Fig. 1B) to a R_a value of 100 nm of the most rough substrate (Fig. 1F). On the other hand, the reference flat Au surface exhibits a R_a value lower than 1 nm (Fig. 1A). AFM line profiles (Fig. 1B-F bottom) of the rough gold substrates confirmed the precise control of the surface morphology, revealing a homogeneous increase of nanoroughness in the different samples. In addition, we fabricated spatially controlled micropatterned surfaces with alternated flat and nanorough gold features, by simply combining the SGDR process with standard lithographic techniques. A representative scheme and a holographic microscopy characterization of such substrates are reported in Fig. 1 (G-H). As clearly shown, spatially confined nanostructured and flat Au stripes (20 μm wide) can be realized.

3 INTERACTION BETWEEN NEURONS AND NANOSTRUCTURED SURFACES

In this section, we discuss how surface nanoroughness influences the biological response of neurons, namely cell adhesion, morphology, differentiation, and cell survival. We studied SH-SY5Y cells as a model system for their particular sensitivity to environmental stimuli and for the importance of functional biomaterials in neural research. The first investigation was directed to estimate cell adhesion onto the different nanotopographies. Fig. 2A reveals a significant decrease of cell attachment on the nanostructured substrates, directly related to the nanoroughness level. Interestingly, small differences in R_a are clearly sensed by the cells which exhibits different adhesion capability. At the highest value of R_a we observed a dramatic decrease of cell adhesion, down to 10-15% of the control flat substrate. We thus explored cell fate after interaction and adhesion onto such nanotopographies. We performed a test capable to distinguish between apoptotic and necrotic cells, based on *in vivo* staining with FITC-conjugated annexinV and propidium iodide (PI). Fig. 2B elucidates the dependence of cell fate as a function of nanoroughness: the substrate nanostructuration, besides hindering cell adhesion, strongly elicits necrosis processes with a trend directly related to the R_a value. Noteworthy, even in the substrate with the minimal R_a value (36 nm), *c.a.* 50% of adherent cells undergo necrosis processes. In the other more rough substrates, cell necrosis approaches 90-95%. On the other hand, no pathways of programmed cell death were induced by nanostructuration. Confocal imaging of SH-SY5Y cells cultured onto flat (Fig. 2C) and nanorough (Fig. 2D) substrates revealed that flat gold surface allows specific cell adhesion, axonal autogrowth, functional cytoskeletal orientation, and nucleus integrity. On the other side, the few cells grown onto the nanorough substrates showed a round shape, a tendency to form clusters, and lack of specialized structures. The nucleus results condensed, suggesting that these cells are undergoing necrosis. Moreover, SH-SY5Y cells cultured onto flat surfaces

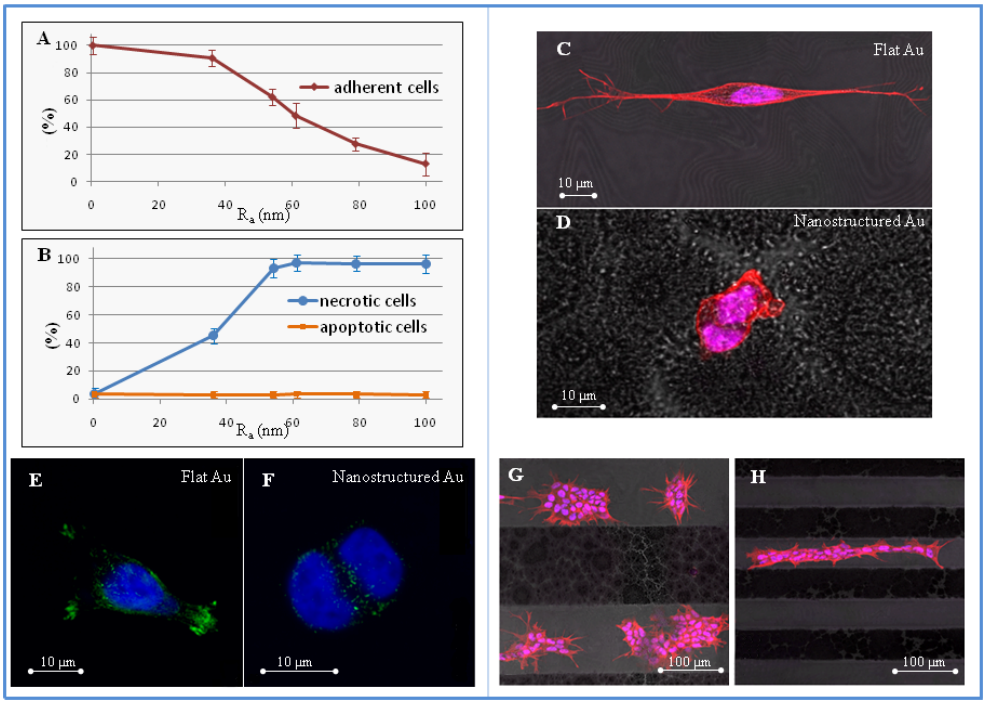

Figure 2: (A) SH-SY5Y cell adhesion onto different nanostructured gold surfaces with increasing values of nanoroughness. (B) Necrotic/apoptotic cells as a function of nanoroughness. (C, D) Representative confocal images of SH-SY5Y cells cultured onto (C) flat and (D) nanorough (R_a = 100 nm) surfaces. (E, F) Confocal images of a correct adhesion complex pattern (E, namely neurons onto flat Au) and of a not organized distribution of vinculin plaques (F, namely neurons onto rough Au). (G, H) Confocal images showing the selective adhesion of SH-SY5Y cultured onto flat/nanorough micropatterned stripes.

(Fig. 2E) presented a clear and correct pattern of adhesion complexes, identified as green spots (vinculin aggregates) that are mainly localized at the periphery of the cell. On the other hand, cells seeded onto the rough substrates (Fig. 2F) showed a sporadic and not organized distribution of vinculin plaques, indicating poor, disorganized and not stable focal adhesion complexes. As a final point, we demonstrated the possibility to control cell adhesion and proliferation onto micropatterned substrates with alternated flat and nanorough surfaces, showing that the control of such nanotopographies can guide substrates toward cytophilic or cytophobic behavior. In this respect, neurons cultured onto 100 µm (Fig. 6G) or 50 µm (Fig. 6H) of flat/nanorough patterned stripes show, in fact, selective growth onto the flat stripes, in close agreement with the results presented above. Therefore, by only varying the surface nanostructuration, neuronal cell adhesion and growth can be easily and precisely controlled.

4 IMPACT OF NANOSCALE ROUGHNESS ON BACTERIA

Surface nanoroughness can also influence the biological response of bacteria in terms of cell morphology as well as variation of their gene expression and proteomic profile. In particular, as shown in Fig. 3A-B, we observed a clear change in the cell morphology of the bacteria grown on the nanorough substrates with respect to flat gold surfaces, namely the absence of the adhesive organelles type-1 fimbriae. We quantified by RT-qPCR the expression of genes used in the production of fimbriae, upon interaction and adhesion on such nanotopographies. The differential expression of type-1 fimbriae, known as "phase variation", is associated with the inversion of a short element of DNA that is situated in the *fim* gene cluster [6]. This cluster contains a promoter which directs the transcription of all the fimbrial structural genes in one but not in the other orientation (*fim* operon in "ON" or "OFF" orientation, respectively) resulting in "all-or-nothing" expression level (Fig. 3C) [11]. In our experiments, a significant difference of mRNA expression level was found in the regulation of the *fim* operon in the bacteria grown on the nanorough surfaces as compared to the control flat one. In particular, the expression level of the fimbrial structural component *fimA* and *fimI* were approximately 57±3% and 75±5% for bacteria grown on the nanorough substrates (Fig. 3D), while the expression level of *lrhA*, which encodes the repressor of the *fim* operon, is 127±6% for bacteria grown over rough substrates (Fig. 3D). To the best of our knowledge, this is the first time that a pure nanotopographical cue was found to induce the loss of fimbriae. These remarkable observations led us to investigate the proteomic profile of

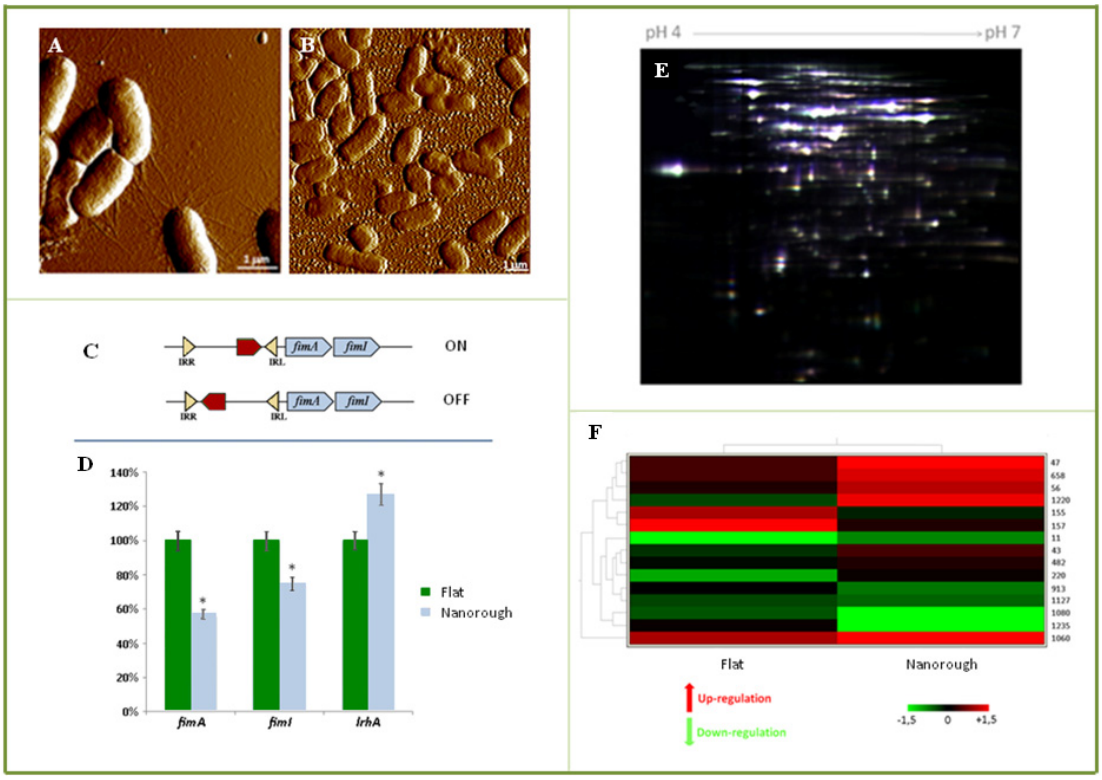

Figure 3: Representative AFM pictures of *E. coli* cells growing onto flat (A) and nanostructured (B) gold substrates. The lack of fimbriae is evident. (C) fimbrial operone mechanism and (D) RT-qPCR analyses of bacteria growing onto flat and nanorough samples. (E) Representative 2D-DIGE gel and Extended Data Analyses (F) with up/down regulated proteins.

E. coli, upon interaction with the nanorough substrates, by the 2D-DIGE proteomic technique. Fig. 3E shows a representative 2D gel in which ~1500 protein spots of *E. coli* grown on both glass and nanorough gold substrates were identified. Among the ~1500 protein spots in the 2D gel, we identified 15 of them which showed statistically significant up and down trends of regulation, shifting from flat to nanorough surfaces, as shown by the different chromatic scales in the Extended Data Analysis (EDA) displayed in Fig. 3F. Such identified proteins are involved in protein biosynthesis, protein transport, metabolic pathway and DNA repair system, demonstrating that nanotopography may directly and significantly affect the biological response/function of bacterial cells.

5 CONCLUSIONS

Our results demonstrated that both neurons and bacteria cells have a surprisingly high sensitivity to small variations in nanoscale features. Regarding SH-SY5Y cells, by properly controlling the surface roughness, we were able to realize cytophilic or cytophobic surfaces, useful in the development of biomaterials for neural research. On the other hand, nanostructured gold substrates induced the loss of type-1 fimbriae in adherent *E. coli*, along with dramatic changes in the genomic and proteomic profiles. Our data highlighted two important issues: (*i*) even an apparently weak physical stimulus, *i.e.*, a nanoscale variation of surface topography, may cause important responses in neurons and bacteria cells; (*ii*) in addition to physico-chemical investigations, deep biochemical and molecular biology approaches are very important to study the interaction processes of cells with inorganic surfaces.

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