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Inclusion of Gold Nanoparticles in Meso-Porous Silicon for the SERS Analysis of Cell Adhesion on Nano-Structured Surfaces

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Abstract

The study and the comprehension of the mechanism of cell adhesion and cell interaction with a substrate is a key point when biology and medicine meet engineering. This is the case of several biomedical applications, from regenerative medicine and tissue engineering to lab on chip and many others, in which the realization of the appropriate artificial surface allows the control of cell adhesion and proliferation.

In this context, we aimed to design and develop a fabrication method of mesoporous (MeP) silicon substrates, doped with gold nanoparticles, in which we combine the capability of porous surfaces to support cell adhesion with the SERS capabilities of gold nanoparticles, to understand the chemical mechanisms of cell/surface interaction.

MeP Si surfaces were realized by anodization of a Si wafer, creating the device for cells adhesion and growth. Gold nanoparticles were deposited on porous silicon by an electroless technique. We thus obtained devices with superior SERS capabilities, whereby cell activity may be controlled using Raman spectroscopy. MCF-7 breast cancer cells were cultured on the described substrates and SERS maps revealing the different expression and distribution of adhesion molecules were obtained by Raman spectroscopic analyses.

1. Introduction

Cell adhesion is at the backbone of a number of biological processes and its correct understanding may accelerate the design of artificial surfaces for applications in tissue engineering, regenerative medicine, personalized lab-on-a-chips, anti-bacterial surfaces, implantable orthopedic or dental devices [1-4]. Cell adhesion is mediated by both specific and non-specific interactions where the former are associated with the formation of specific molecular bonds (ligand–receptor bonds), while the latter are governed by interfacial long and short ranged forces [5]. The formation of specific molecular bonds is the result of a cascade of signals which may be either physical and chemical and these represent the information exchanged between the cell and the substrate. A similar information is modulated by transmembrane adhesion receptors of the integrin family, with dimensions ranging from 30 nm for focal complexes [6] to micro-meters or more for focal adhesions [7]. Focal complexes and focal adhesions mechanically link the extracellular matrix with the termini of actin bundles[8] and are inherently sensitive to the biochemical characteristics, rigidity and spatial organization of the cell/substrate interface[9, 10]. Interestingly, the characteristic length scale of integrins falls in the same dimensional range of nanotechnologies. On reproducing and expanding the complex architecture of the extra cellular matrix with details over multiple scales, nanotechnology may provide instruments to understand how the cellular sensory machinery interacts with extremely small cues to regulate cell behavior and adhesion. Porous silicon (PSi) is a biomaterial in which conventional silicon is modified through electrochemical procedures to contain a layer of pores within its structure[3, 11]. The artificially introduced network of pores may vary in size and shape to a large extent and the distribution of these in the porous matrix can be finely adjusted on changing few parameters of the fabrication process, including etching time, current intensity, active etchant concentration, temperature of the process[3, 11]. PSi substrates with a fixed[3, 12] or smoothly variable pore size[13] were used to verify cell adhesion as a function of surface topography. Recalling that PSi surfaces are divided into micro-porous (MiP), meso-porous (MeP) and macro-porous (MaP) silicon depending on the pore size S that transitions from $S < 2\ nm$, to $2 < S < 50\ nm$, to $S > 50\ nm$ for the MiP, MeP and MaP architectures[14], results presented in[3, 13] indicate that nano-scale surface topography with feature sizes in the low MeP regime accelerate cell growth and adhesion.

Here, we demonstrate the fabrication of MeP Si substrates in which the average pore size is controlled in the $10 - 20\ nm$ range with a large fractal dimension $D \sim 2.8$. Electroless deposition techniques [15, 16] were used to incorporate networks of gold nano-particles in the porous surfaces, which impart to them superior SERS capabilities. MCF-7 breast cancer cells were

cultured on the described substrates and SERS maps were collected revealing the different expression and distribution of adhesion molecules on nanostructured substrates in comparison to MeP samples not decorated with gold.

2. Materials and methods

Hydrofluoric acid (HF) and ethanol were purchased from Sigma. Deionized water was used for all experiments. All chemicals, unless mentioned otherwise, were of analytical grade and were used as received.

2.1 Fabrication of porous silicon substrates

Meso-porous silicon is a form of silicon containing a network of nano-pores in its structure, in which the average pore size is controlled in the nanometer rante. To fabricate the devices we used the methods described in [3, 11, 12, 17]. Meso-porous silicon was obtained by anodization of boron-doped silicon wafer (100) with resistivity 5-10 Ω-cm, using an electrolyte mixture of hydrofluoric acid (25%), water (25%), and ethanol (50%). A constant current density of 10 mA/cm^2 was applied to the electrolytic cell for 5 min at 25°C. Sample was rinsed in deionized water, then in ethanol and at the end in pentane to completely remove any water and chemicals residues from the porous surface.

2.2 Gold electroless deposition

Electroless deposition is a technique in which metal ions in solution can be reduced and deposited as metals using appropriate reducing agents, in presence of a catalyst that can accelerate the electroless reaction allowing for the oxidation of the reducing agent. Electroless deposition has been widely explored in the fabrication of silver nanoparticles [15-17]. In Reference [18], silver nanoparticles have been deposited on a micro/structured, super-hydrophobic surface for the detection of few molecules. Here, we revise the method to exploit the synthesis of gold particles on porous silicon surfaces.

Porous silicon samples were dipped in 10 ml of a water solution containing HF (C=0.15 M) and Gold (III) Chloride ($AuCl_3$) (C=1mM) for 3 min. In the experiments, the temperature of the system was maintained fixed as T=50 °C. In the solution, a redox reaction occurs between gold ions (oxidizing species) and the porous silicon substrate (reducing species), yielding gold nano-spheres with an average diameter lower than 20 nm. The particles were deposited both on the porous surface and into the pores. The oxidation of Si produces the electrons which are necessary for the metal reduction. The device is then washed with water at room temperature to block the reaction.

2.3. SEM analysis

SEM images of the substrates were taken using a dual beam (SEM-FIB) – FEI Nova 600 NanoLab system with a beam energies of 15 kV and electron currents of 0.14 nA.

2.4 MCF7 culture on the substrates

MCF-7 cells from breast carcinoma were chosen to test the ability of the porous substrate in promoting cells' growth. The culture medium used was a Dulbecco's modified eagle's medium (DMEM, Euro-clone) with addition of 10 % heat-inactivated fetal bovine serum (Euroclone), streptomycin (0.2 mg ml^{-1}) and penicillin(200 IU ml^{-1}). Cells were incubated in a Petri dish at 37°C with 5% CO_2 in humidified environment, until they reached 90 % of confluence. At this point, the medium was removed and cells were detached by trypsinization, with a solution of 0.25 % Trypsin-0.53 mM EDTA (Euroclone) for about 5 min at 37 °C. Trypsin was then deactivated by addition of medium and then removed by centrifugation of cells suspension at 1300 rpm, 5min, 18 °C. The recovered cells were plated on the porous silicon substrates with and without gold nanoparticles with a confluence of 50%. Cells, after washing in phosphate buffered saline (PBS) with calcium and magnesium, were fixed with formalin 2% for 40 min at 37°C and rinsed newly with PBS, for analysis by Raman spectroscopy.

2.5 MCF7 Raman spectroscopy analyses

SERS measurements were performed by an InVia Raman microscope from Renishaw Ltd, working in backscattering configuration with a 633 nm laser source, focused on the sample with a 50x/0.75NA objective. The signal was analysed with a 1800 lines/mm grating, ensuring a spectral resolution of 1 cm^{-1}. Laser power used at the sample level was maintained in the very low range of 12 μW to avoid cell damage. In SERS mapping measurements, pixel size was fixed as 1 μm and integration time as 3 s. Cells cultured on porous silicon without gold were also analysed by Raman spectroscopy as a comparison. In conventional Raman measurements a power of 10 mW was used to obtain well defined spectra.

3. Results and discussion

3.1. SEM analysis

Mesoporous silicon surfaces were realized by anodization of silicon, following the methods described above. Two typologies of samples were used: (i) simple meso-porous silicon without the inclusion of Au nanoparticles and (ii) meso-porous silicon with Au nanoparticles in its structure, with improved SERS abilities. Metal deposition was realized by electroless (see

methods) and both samples were characterized using SEM. **Figure 1** shows SEM micrographs of mesoporous silicon surfaces before (**a**) and after (**b**) gold deposition. All the samples show a porosity where the average pore size is around 20 nm. After electroless deposition, samples are covered by gold nanoparticles with a diameter smaller than 20 nm and they appear uniformly distributed on the porous surface (**figure 1c,d**).

3.2 MCF7 culture on the substrates

MCF-7 breast cancer cells were cultured on MeP Si substrates with and without gold as described in the Methods. Cells show to interact with the porous surface and their organization on the substrate is described by an uniform distribution for both the samples (i) without and (ii) with the gold nanoparticles, with no or minimally appreciable differences among the two, as it is visible from optical images in **figure 2a and c,** this indicates that gold does not affect cell behaviour and cell-surface interaction.

3.3 MCF7 Raman spectroscopy analyses

Raman micro-spectroscopy was performed on MCF-7 cells cultured on the two kinds of substrates (mesoporous Si and mesoporous Si/gold nanoparticles). The insets in the optical image of **figure 2a** and **c** indicate the area where Raman mapping was performed for each sample. The Raman mapping results are reported in the zoomed images of **figures 2b and d.** The maps are contour maps proportional to the intensity of the CH vibration, observed at 1445 cm-1. In conventional Raman, this peak is one of the more intense, moreover, it is an index of protein CH deformations vibration in deoxyribose [19]. The spectra of the cells are clearly visible in all the points of the two maps. The two maps show noticeable differences, the first, is the laser power that one needs to adjust for the acquisition: without gold nanoparticles, Raman spectra are obtained setting a laser power as large as approximately 10mW; differently, when gold is deposited on the substrates, the laser power necessary to reach comparable counts in the Raman spectra is roughly 12 μW, that is, 3 orders of magnitude less than for conventional porous substrates. Moreover, the two maps are completely different (figure 2b and d): in absence of gold we have an uniform map in which the intensity of the colour varies proportionally to cell thickness. When gold nanoparticles are deposited on the porous surface, the change in colour is not proportional to cell thickness but it is differently distributed, and this may instead reflect a change in the chemical composition of the cell on the substrate. These data become more clear observing the collected spectra (**figure 3**). When cells are analysed by conventional Raman, each point of the map shows the same spectrum characterized by the principal peaks typical of cellular matter (the Amide III spectral feature, between 1240 and 1350 cm^{-1}, the CH vibration, 1445 cm^{-}

[1], the Amide I band sensitive to protein secondary structure, 1640–1685 cm^{-1}), as evident in figure 3a. On the contrary, SERS analysis of the MCF-7, obtained from the samples with gold NPs, reveals different spectra (different colors in the map), supporting the possibility to individuate expression and distribution of adhesion molecules on the MeP substrates (figure 3b).

Specifically spectra of figure 3b show some peaks attributable, for example, to integrins or other proteins, which can indicate the adhesion of the cells on the substrate. A summary of some peaks assignments associated to these proteins is displayed in Table 1. In particular, peaks which can be attributed to amino acids with aromatic rings, such as phenylalanine, tryptophan, and tyrosine, give strong SERS signals at 1564 cm^{-1}, 1515 cm^{-1}, 1352 cm^{-1}, 1175 cm^{-1} with slight shifts in frequencies respect to those reported in literature, due to the specific cell/surface interactions. Another amino acid that also contributes with a strong SERS signal is histidine, which has two alternating double bonds in the imidazole ring giving vibrations close to the aromatic amino acids (1569 cm^{-1}, 1359 cm^{-1}) [20-23].

Spectra show also (i) a peak at 1618 cm^{-1}, indicative of phenylalanine or tyrosine [20], but also due to the contribute of amide I band [24]. Amide III is also evident through peaks at (ii) 1352 cm^{-1}: it is from the amide III band and indicates the presence of α-helix [20-22]. Another peak at (iii) 1250-84 cm^{-1} reinforces the evidence of the amide III band [25, 26]. Finally, (iv) the peak at 1100 cm^{-1} indicates the vibration of C-N in the peptide bond [22].

4. Conclusions

MeP Si substrates were realized by an anodization method, with an average pore size in the $10-20\ nm$ range and a large fractal dimension $D\sim2.8$. The described porous surfaces were designed to favorite cellular culture, because (i) of a characteristic length scale that promotes adhesion, and (ii) the possibility to incorporate, in the pores, nutrients and grow factors that can accelerate cell proliferation. In this work we revise similar porous substrates to incorporate gold nanoparticles which permit to interrogate cell response using SERS. Gold nanoparticles were deposited using electroless techniques; the consequent SERS effect makes visible the molecules responsible for the cells/surface interaction. MCF-7 breast cancer cells were cultured on the described substrates and SERS maps were acquired by Raman spectroscopy. The spectra

obtained for each point of the maps reveal the presence of proteins which could be related to different expression and distribution of adhesion molecules on the nanostructured substrates; differently from the spectra obtained from conventional Raman which show the principal peaks of the cells solely. This analysis may yield to the creation of a database where the Raman signature of adhesion molecules in cells challenged with different patterns is compartmentalized as a function of pattern nano-topography. This information may support engineers and scientists in the rational design of bio-mimetic surfaces.

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Figure/Table Captions

Figure 1 SEM micrographs of MeP Si substrates: a) and b) in absence of Au nanoparticles; c) and d) with clusters of Au nanoparticles.

Figure 2 MCF-7 culture on the mesoporous silicon and Raman analysis: a) and c) optical images of MeP Si substrates, with and without gold, after cell culture; b) and d) Raman mapping of the samples respectively with and without gold.

Figure 3 Raman spectra of MCF-7 cells cultured: a) on a mesoporous silicon sample and b) on a mesoporous silicon sample decorated with gold nanoparticles.

Table 1 Peaks assignments as reported in literature associated to proteins of adhesion.

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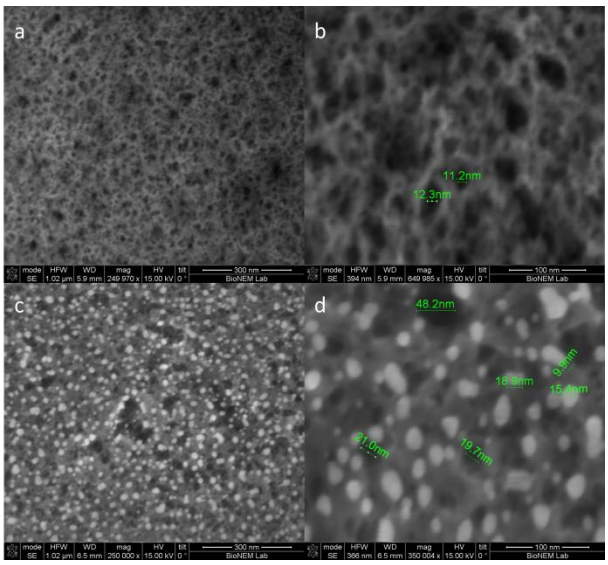


Figure 1

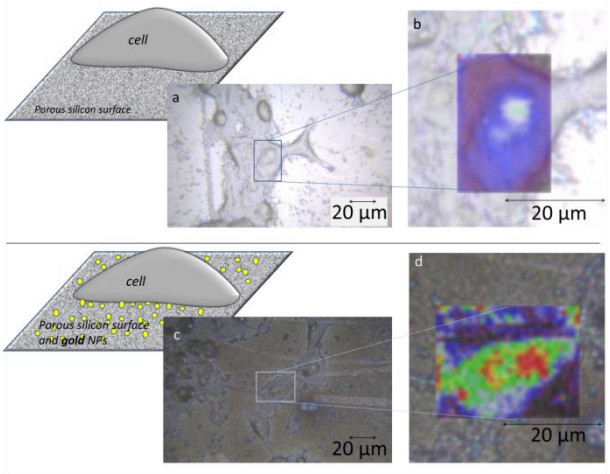


Figure 2

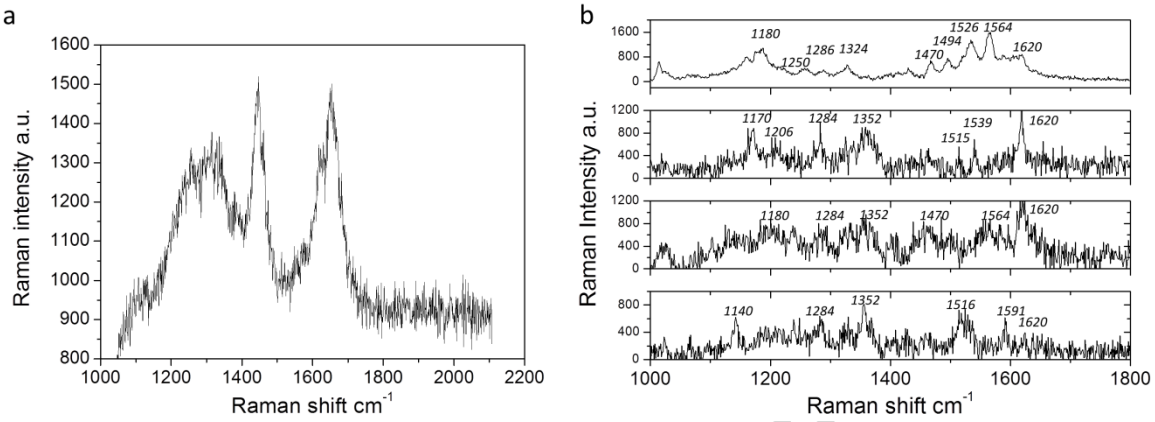
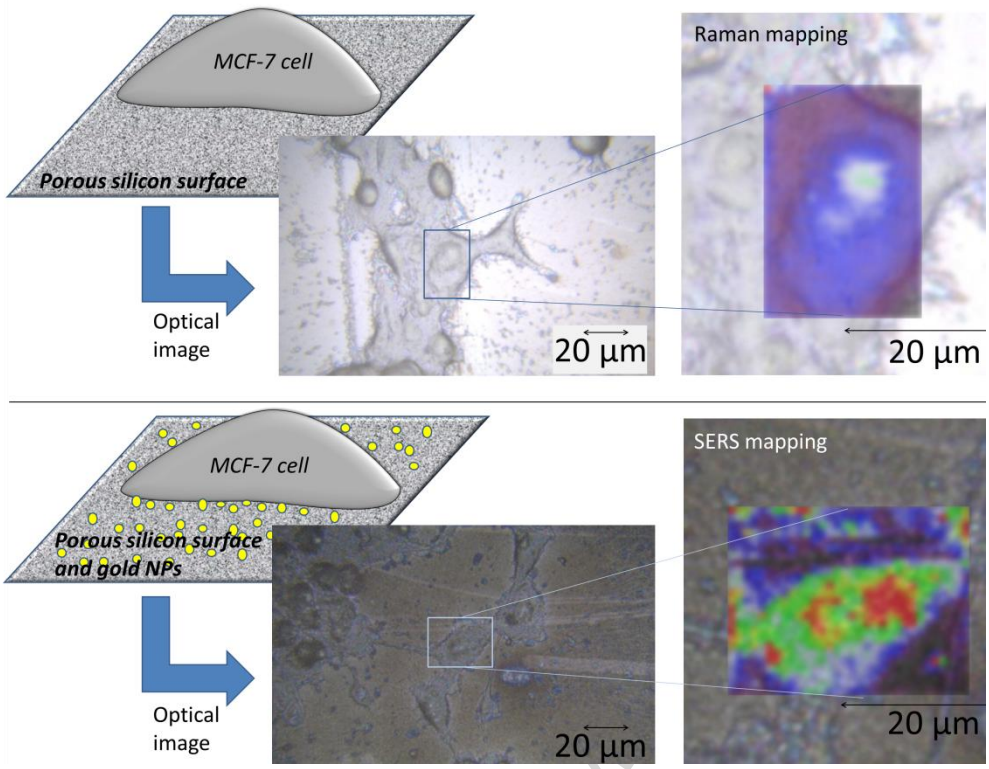


Figure 3

Table 1

SERS analysis of the MCF-7 and principal peaks assignments

Frequency (cm^{-1})	Assignment
1618-1620	Phenylalanine or Tyrosine Residues/Amide I [20-24]
1564-9	Tryptophan or Histidine Residues [20-23]
1515	Phenylalanine, Tyrosine, or Histidine Residues [20-23]
1352-9	Tryptophan Residue [17-20] and Amide III Band [20-22]
1250-84	Amide III Band of (α-helix and random coil) [25,26]
1170-80	Phenylalanine or Tyrosine Residues [25,26]
1100	(C-N) peptide bond stretch vibration [22]



Graphical Abstract

Highlights

1) designing and developing of a fabrication method of mesoporous (MeP) silicon substrates decorated with gold nanoparticles;
2) these devices are able to support cell adhesion and proliferation and simultaneously they works as SERS substrates;
3) SERS Raman analysis of cells cultured on these substrates provide indication about cells adhesion degree and cells behaviour on them.