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Letters

AZO-POLYSILOXANES AS NEW SUPPORTS FOR CELL CULTURES

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Abstract

The paper introduces a new class of materials with azo-polysiloxanic structure bearing the property to generate nano-structured surfaces by laser irradiation. The ability to modulate the optical response of the film, through a modification of the polymer chemical structure, has been investigated. The azo-materials were tested for their ability to support cell adhesion and growth, with very promising results. A future use of these materials as growth support in cell cultures is of great interest, due to an easy, one step-method to generate the surface relief grating and to the possibility to introduce a large range of chemical modifications due to the presence of the chlorobenzyl groups in the polymeric side-chain.

Keywords: azo-polysiloxanes, cell cultures, laser nano-structuration, surface relief gratings.

Introduction

A tissue architecture, integrity and function are tightly dependent on the communication between the component cells and their micro-environment. In most cases, this consists of a heterogeneous, macromolecular matrix that is specific to a given tissue. The relationship between the cells and the extracellular matrix (ECM) is dynamic and reciprocal, changing with cellular differentiation or tissue remodeling. This interplay has become a dominant theme of basic biomedical research within the last years. One key issue of this communication is the transformation of micro-mechanical information provided by the ECM into biochemical signals which regulate cellular processes, such as adhesion, differentiation, proliferation and migration. Since the early developmental phases, embryonic cells produce their own extracellular scaffolds, by secreting many types of molecules in the surrounding space, according to a well-defined program of differentiation (Adams and Watt, 1993; Vaino and Muller, 1997). The different spatial organization of these secreted molecules gives rise to a great variety of natural scaffolds in which cells continue to proliferate and to organize themselves in order to build tissues and to accomplish their natural functions. This aspect is mostly investigated in vitro, in a non-physiological environment, using flat and rigid substrates for cell culture, which often make the results difficult to interpret [1]. Modifying the cell growth surface, in a controlled manner, using nano-structured synthetic materials can help understanding the complex mechanisms of cell adhesion and migration in normal conditions and different pathologies [2-4]. For example, in the case of cancer tumors, the high rigidity of the ECM can induce an extensive cell spread [5-11].

The influence of the support surface geometry on cell development was intensely studied especially for substrates obtained by micro- or nano-lithography [12-15]. Micro- or nano-structured surfaces were usually produced using lithography techniques relying on multiple technological step procedures [16-18]. A simpler method to obtain nano-structured surfaces in one single step is the laser irradiation of the azo-polymeric films [19-22]. In contrast with classical interference lithography, the method proposed here produces reversible

modifications of the film surfaces and can provide a real-time control of the topography during the cell growth experiment. One of proposed mechanism of the surface relief formation, involves a mass displacement of the material [23], probably relieved by the continuous *trans-cis-trans* conformational changing of the azobenzene groups during laser irradiation that prevent the material stabilization in a solid state [24] and the polymer displacement, in agreement with the cage-breaking model [25]. The use of the azo-polymeric materials as ECM in cell culture brings an advantage when compared to other materials, taking into consideration the possibility to generate a light-controlled mechanical stress at film surface, due to the cis-trans isomerization of the azo groups. In spite of the evident advantages of the azo-polymeric materials, only few reports dedicated to this class of polymers having an azo-polymethacrylate structure have been published so far in the literature [26-29].

In the present paper we propose a new class of biomaterials, bearing the property to generate nano-structured surfaces by laser irradiation. These azo-materials were tested for their ability to support cell adhesion and growth, with very promising results. Moreover, we show that the cells-substrate interactions can be modified as a function of the polymer chemical structure and relief geometry. Further, the presence of very reactive chlorobenzyl groups in the polysiloxane side-chain enables the connection of different chemical structures, making possible to modify the chemical signals transmitted by the azo-polysiloxane to the cellular membrane, on cell adhesion, proliferation and migration properties. This class of azo-materials is also very interesting from the perspective of elucidation of the nano-structuration mechanism, taking into consideration the polymers low Tg values, responsible for a greater mobility of the azo-groups.

Materials and Methods

Methylene chloride (code 270997), dimethylsulfoxide (code 494429), methanol (code 494437), chloroform (code 472476), 4-phenylazophenol (code 131083), 4-trifluoromethyl aniline (code 224936) and 4-nitroaniline (code 185310) were purchased from Aldrich,

Steinheim, Germany and used without supplementary purification. 4-Hydroxy-4'-substituted-azobenzenes were prepared in nearly quantitative yield (85–95%) from the corresponding p-aniline that was diazotated and coupled with phenol according to the procedure reported for the synthesis of the 4-hydroxy-4'-cyanoazobenzene derivative. [30]. The ((chloromethyl) phenylethyl)methyldichlorosillane (AB110958) used to prepare the linear polysiloxane was purchased from ABCR GmbH & Co. KG, Karlsruhe, Germany and used without supplementary purification.

The azo-polymers were prepared starting from a polysiloxane containing chlorobenzyl groups (Reaction Scheme), details concerning the synthesis and characterization being previously reported [31,32]. Different chemical structures of the azo groups were used, as following: 4-phenylazobenzene, 4-((4'-(trifluoromethyl)phenyl)azo) phenol and 4-((4'-(nitro-phenyl)azo) phenol.

The polymeric films were prepared by a spin-coater technique and layered either on glass or poly(methyl methacrylate) (PMMA) support. The glass supports were produced by ISOLAB, Laborgeräte GmbH, Germany and the PMMA one by PERSPEX Distribution Ltd, U.K.

The surfaces were structured using an interference pattern (Figure S1 - Supporting Information) produced by the superposition of two laser beams incident with an angle θ onto the film surface. In this geometrical configuration, the light intensity is modulated sinusoidally along the film surface with a periodicity Λ controlled by the incident beams angle according to the formula:

$$\Lambda = \frac{\lambda_L}{2\sin\theta}$$

where λ_L is the laser beam wavelength.

The beams were delivered by a laser diode at a wavelength of λ_L=488 nm in the optical absorption band of the azobenzene molecule, with a total intensity of 180 mW.cm^{-2}. The beam polarization was set horizontally (p-polarized) for the experiments reported here. The formation of the surface modulations was probed in real time by measuring the evolution of the helium-neon laser beam intensity diffracted in the 1rst order. The wavelength of the

probe was 633 nm, outside the absorption bands of the polymer materials to limit its influence in the film surface patterning process. For bi-dimensional surface gratings, two successive exposures of the film to the interference pattern were performed by rotating the film by 90°. The film topography was characterized by atomic force microscopy (AFM) using the 5100 AFM from Agilent Technologies driven in the contact mode.

Cell adhesion and growth were monitored by immunofluorescence microscopy, at 24 hours post-seeding, using a Nikon Eclipse E600 microscope. Actin filaments and microtubules were visualized with the 60X objective, using Alexa Fluor 488/594-labelled phaloidine and mouse anti-human tubulin antibodies, followed by Alexa Fluor 488/594-labelled goat anti-mouse antibodies, respectively. Cell nuclei were stained with DAPI and visualized with the 20X objective.

Results and Discussion

The azo-polymers were deposed onto a glass or poly (methyl methacrylate) support using a spin-coater technique, followed by irradiation with a polarized laser source. Two types of surface geometry were obtained (gratings or pillars - Figure 1) which were further characterized by AFM.

The main geometric characteristics of the surfaces are the relief periodicity (0.8-2.5 μm) and height (50-200 nm). The laser irradiation system allows the control of both geometric parameters in real time. The characteristics of the grating nano-structured surfaces are presented in Table 1.

An important particularity of the azo-polysiloxanic materials is the low Tg value, situated close to the ambient (30-50^{0}C, as a function of the polymer chemical structure and substitution degree). This is clearly an advantage raising the possibility to study the influence of the ECM rigidity, on cell adhesion and proliferation.

The ability to modulate the optical response of the film, through a modification of the polymer chemical structure, has been investigated by monitoring the deformation properties (modulation amplitude and dynamic) of the films surface exposed to a beam with a sinusoidal

modulated intensity profile. In particular, the incorporation of additional chemical structures onto the azo-polysiloxane chains, which are able to produce physical interactions (π-stacking, H-bonding) within the polymer film, can be used to stabilize the photo induced deformations, besides providing surface functionalization. The modification of the materials chemical structure is an important parameter, impacting the process of surface grating formation. Addition of chemical functions aiming at providing inter- or intra-chain physical interactions is expected to modify the film behavior; this will also result in a decrease of the material reactivity to light, leading to a slower surface modulation, thus directly impacting on the maximum efficiency achievable. In the case of the azo-polysiloxanes with a Tg close to the room temperature, a modification of the material chemical structure strongly affected the flexibility of the chains in the polymer film resulting in significant modifications of the polymer behavior under light exposure (see Figure S2 - Supporting Information).

The fine control over the chemical structure of these azo-polysiloxanes was implemented to generate materials presenting different surface chemical properties and modulations capacities. Besides the potential of this new class of polymers for the application considered here, it is important to underline that the low Tg of the azo-polysiloxanes investigated is also interesting from a more fundamental point of view. The high flexibility of the polysiloxane chains, which can be tuned by chemical engineering, offers a valuable tool to investigate the process of the polymer matrix reorganization under optical excitation, providing new information on the mass transport mechanisms optically induced in the polymers.

The next step of the study addressed the interactions between the nano-structured surfaces and cells. Cytoskeleton structure comprising microtubules and actin filaments was investigated in human hepatoma cells (HepaRG) grown on the azo-polysiloxanic films listed in Table 1 to reveal the shape and basic organization of the cytoplasm, as a measure of cell-substrate interactions. The cell nucleus was also evidenced by specific staining, to determine the total number of viable cells grown on different surfaces.

Taking into consideration that a lower number of cells were able to grow on all structured surfaces containing a pillar relief as compared to the reference, only the grating nano-

structured surfaces will be discussed further. Interestingly, the relief geometry had a strong effect on cell growth in the case of Sample 1, a higher number of cell being evidenced on the structured area compared with the plane one (Fig. 2 a, b, c, d). Similarly, a higher (but no so evident) number of cells have grown on the structured area of Sample 2, compared to the plane surface (see Figure S3 - Supporting Information). It is important to note that the cytoskeleton structure, as evidenced by fluorescence microscopy, showed a normal distribution of microtubules and actin filaments throughout the cytoplasm, in these samples, indicating a good adherence of the cells to these substrates. The relief geometry had however no influence on cell growth in the case of Sample 3 and did not improve the cell adherence properties of this particular polymer. As shown in Figure 2 (e, f, g, h) both, the plane and nano-structured surfaces accommodated a similar number of cells with a rather disorganized cytoskeleton. Taken together, these observations lead to the conclusion that the chemical structure of the polysiloxanic films has a strong influence on cell-substrate interactions, influencing the division rate and adherence.

An intriguing behavior was observed for Sample 3, as a consequence of the support used to deposit the film. Thus, when a poly (methyl methacrylate) (PMMA) support was employed, a very low number of cells were able to grow on the plane area compared to control, while no cells were identified on the structured surface (Figure 3). Moreover, unlike the control (Figure 3b), the cytoskeleton elements were organized in aberrant structures (Figure 3d). A possible explanation for this striking difference is that the interaction between the support and the azo-polysiloxanic film that could induce supramolecular ordering differences at the film surface. Considering the polymeric film thickness (500 nm), these influences can be significant. Unfortunately, because of problems regarding the polymer adherence to the PMMA support, this type of investigation was possible only for Sample 3.

As Sample 1 showed the best results in supporting cell growth, this polymer was further considered for a study concerning the influence of the relief periodicity. As shown in Fig. S4

and S5 (Supporting Information) the diminution of the distance between the fringes of the gratings results in a lower number of cells adhering to the structured area.

Conclusions

The major conclusion of this study is that the new class of azo-polysiloxanes shown here is capable to generate stable nano-structured films, as a function of the chemical structure and substitution degree. A future use of these materials as growth support in cell cultures is of great interest, bringing two major advantages when compared to the classical materials used so far: a) an easy, one step-method to induce the surface relief grating and b) the possibility to introduce a large range of chemical modifications due to the presence of the chlorobenzyl groups in the polymeric side-chain. Cells are very sensitive to the chemical and physical properties of the polymeric substrate, which they can integrate and transfer to signaling pathways, with immediate consequences on cell fate. Biomaterials are frequently functionalized with various molecules to investigate this relationship, thus the more versatile this derivation is, the larger the range of potential applications. The best results were obtained on the structured surfaces area for Sample 1. Modulating further this surface in a tightly controlled manner, at micrometer and nanometer scale is of particular interest in defining new strategies to design synthetic ECM or biomaterials which may help understanding the molecular mechanism underlying the complex cell- ECM relationship.

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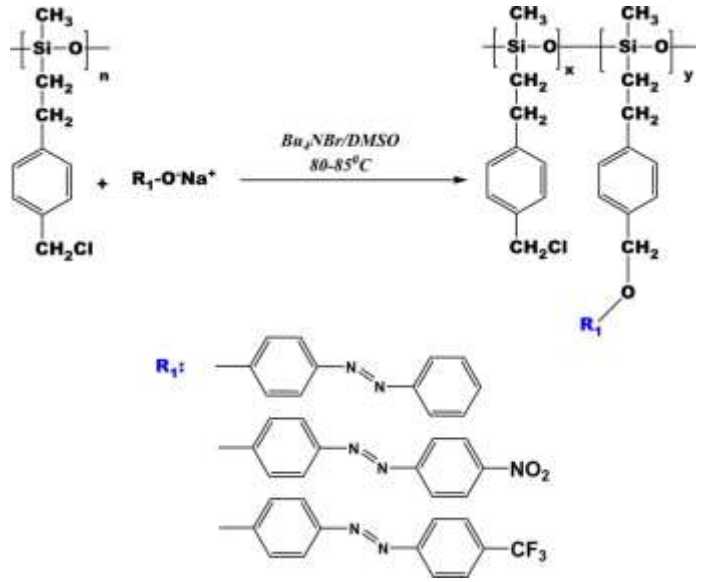

Reaction Scheme

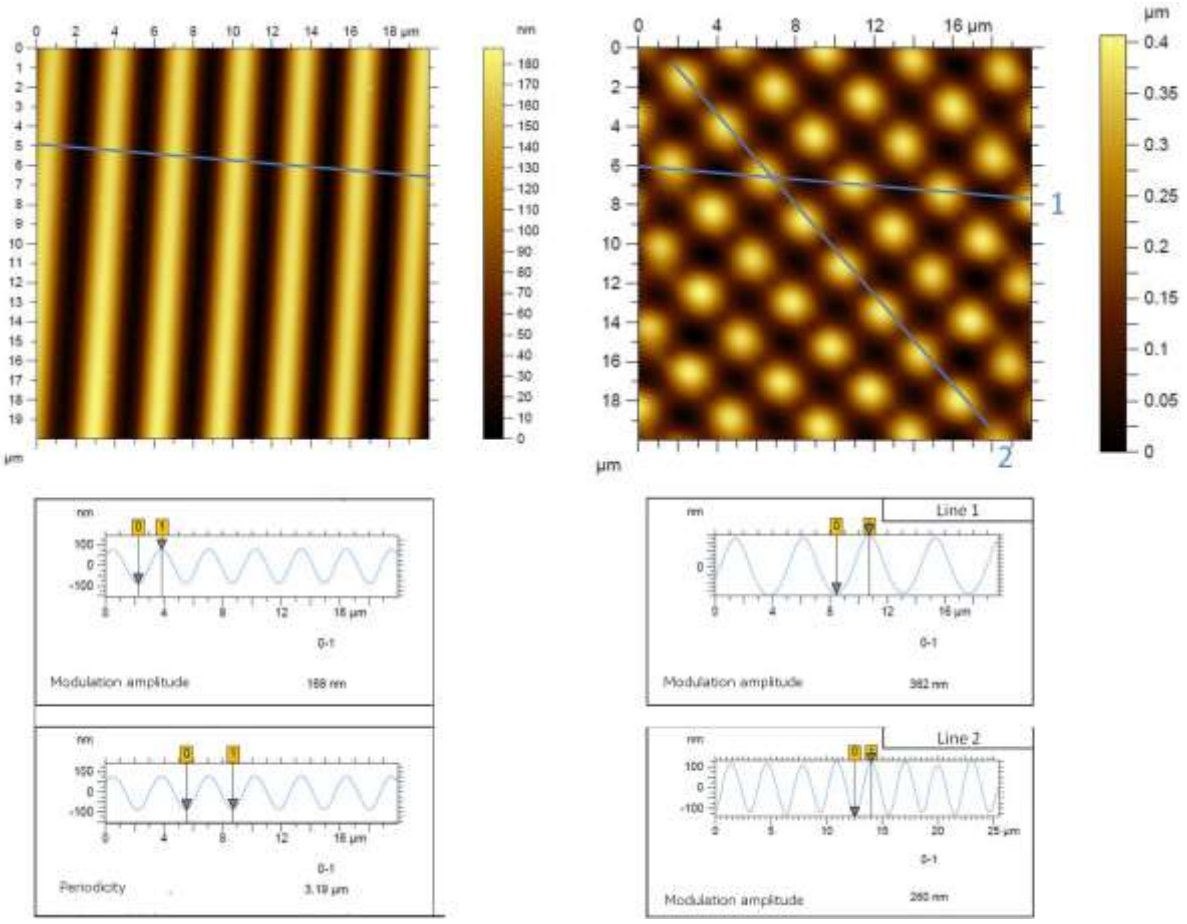

Figure 1. AFM images of azo-polymeric materials having gratings (left) or pillars (right) on the surface

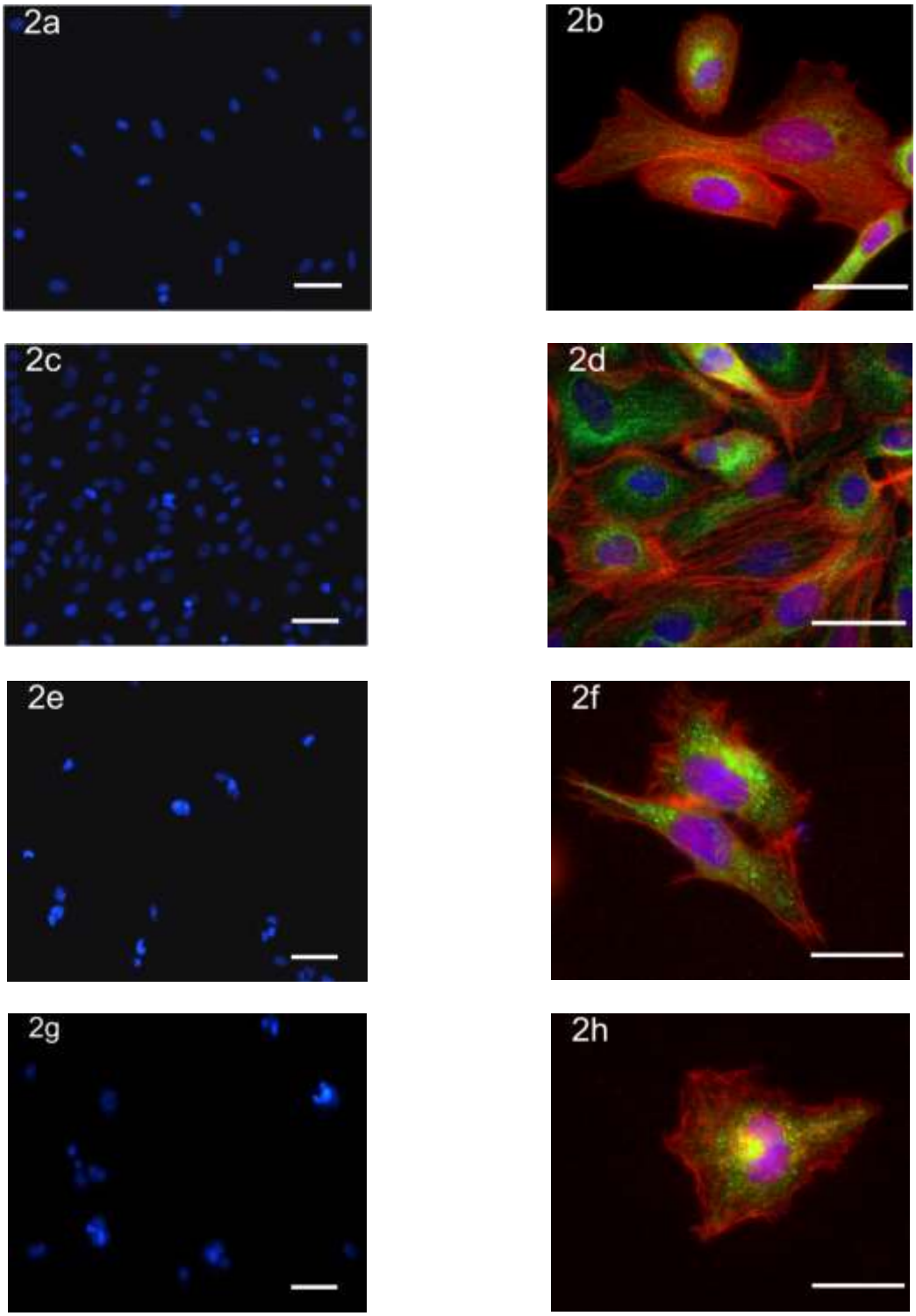

Figure 2. Immunofluorescence microscopy images corresponding to Sample 1 (layered on glass substrate) showing the cell nuclei (2 a, 2c) and the cytoskeleton elements organization, actin -red and microtubules-green (2b, 2d), on the plane (2a, 2b) and structured (2c, 2d) areas and Sample 3 (layered on glass substrate) showing the cell nuclei (2e, 2g) and the cytoskeleton elements organization, actin -red and microtubules-green (2f, 2h), on the plane (2e, 2f) and structured (2g, 2h) areas.

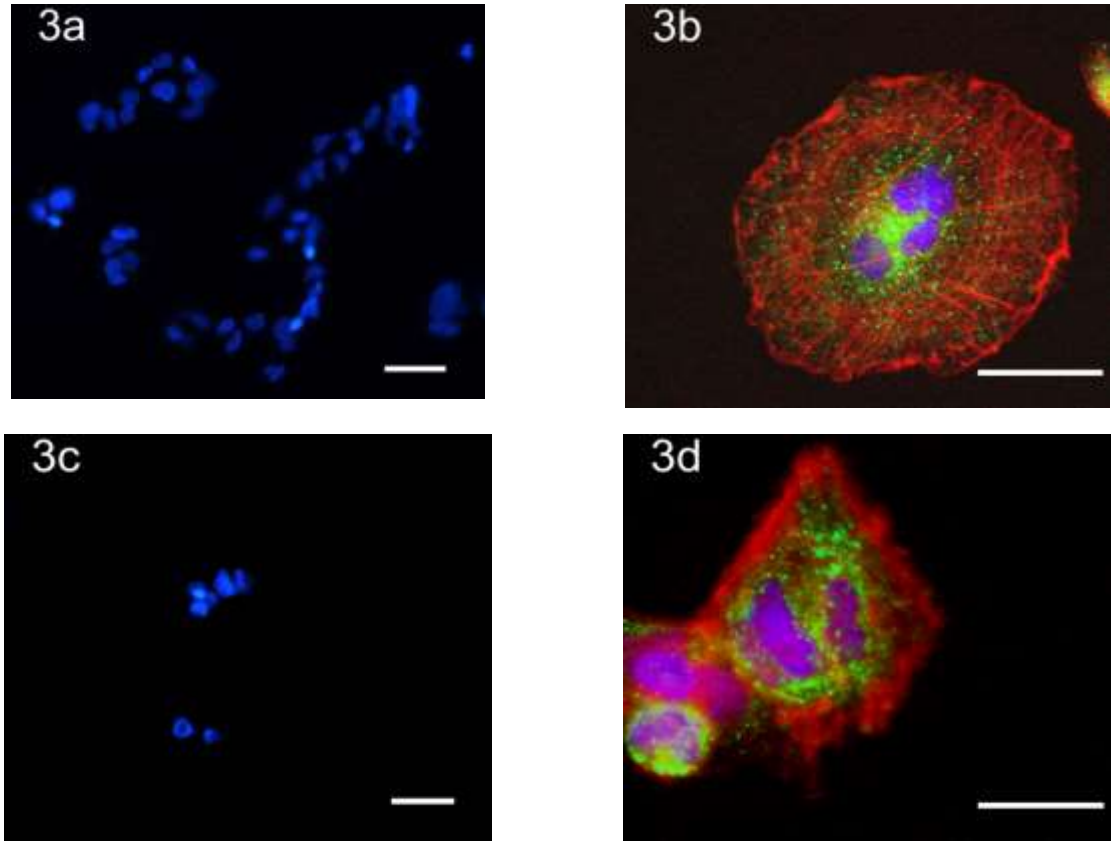

Figure 3. Immunofluorescence microscopy images corresponding to Sample 3 (layered on PMMA substrate; grating periodicity 2.7 μm; relief high 100 nm) showing the cell nuclei (3a, 3c) and the cytoskeleton elements organization: actin -red and microtubules-green (3b, 3d), on a control surface (3a, 3b); and the plane area of the polymer (3c, 3d); no cells were evidenced on the structured area.

Table 1. Geometric characteristics of the grating nano-structured azo-polysiloxane films

Sample code	Azo-group substituent	Substitution degree [%]	Tg [°C]	Relief periodicity [μm]	Relief high [nm]
1	p-NO_2-azobenzene	89	50	2.7	100
2	p-CF_3-azobenzene	92	48	2.7	200
3	azobenzene	94	31	2.7	100
4	p-NO_2-azobenzene	89	50	1.8	60
5	p-NO_2-azobenzene	89	50	0.8	80