Cells behavior on argon plasma treated pNIPAAm thin films

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In the field of bioengineering, various material surface modification strategies were developed in order to favor or preventing the attachment of various biological compounds to the biomaterial. The present contribution reports on the use of specific surface property of a thermo- responsive polymer poly (N-isopropylacrylamide) pNIPAAm obtained by spin coating in combination with argon plasma treatment for tuning cells behavior on treated polymeric surfaces. Topographical information for the plasma treated pNIPAAm coatings obtained by Atomic Force Microscopy (AFM) measurements indicated that RF argon plasma induce surface thinning and pores due to the etching effect of Ar ions present in the discharge. FTIR spectroscopy revealed lower intensity for the specific bonds of the pNIPAAm material and the presence of polar groups favoring enhanced wettability of the surface upon plasma treatment. The biological investigations showed that L929 fibroblast cells proliferation strongly depend on the plasma treatment time, longer treatment time inducing a clear modification of the cells morphology.

Keywords: pNIPAAm, plasma treatment, Cytocompatibility; cell detachment

1. Introduction

The development of polymeric materials whose surface properties can be dynamically tuned by external stimuli in order to ensure additional functionality to the material is of great importance for bioengineering and biotechnology applications [1]. Such materials are often called "stimuli-responsive polymers or "smart polymers (SP)" or "intelligent polymers" [2]. Stimuli can be either chemical (e. g. pH, ionic factors and chemical agents) or physical, such as, temperature, light, and magnetic field [3]. For biomedical applications, the systems that are sensitive to temperature or pH are the most important due to the polymer-polymer and polymer-solvent interactions which indicate a sudden readjustment for small ranges of pH or temperature modifications [4]. Moreover, temperature is the most widely used stimulus for "smart polymers" due to the easiness of the control of this and for the facile applications *in vitro* and *in vivo* [5]. Among so called smart materials, poly(N-isopropyl acrylamide) (pNIPAAM) is of special interest in the field of bioengineering due to the phase change that it undergoes in a physiologically relevant temperature range which favor the cell or protein release [6]. The lower critical solution temperature (LCST) for pNIPAAm is 32°C in water, having a collapsed hydrophobic state above this temperature and an extended hydrophilic state below LCST [7].

An optimal pNIPAAm layer for cell seeding involves complex and non-easily fabrication methods (e. g covalently graft pNIPAAm chains by electron beam irradiation) [8]. Spin-coating technique represent a simpler and more efficient method for manufacture of pNIPAAm layers, which can serve as a thermoresponsive surfaces for cell culture and cell sheet harvesting [9]. The control of thickness and porosity of the pNIPAAm layers play an important role in temperature-induced hydrophilic/hydrophobic state and cell attachment above LCST and detachment below LCST [10]. In this study we report on the argon plasma treatment of pNIPAAM spin-coated thin films in order to modulate the material surface for tuning cell behavior on plasma functionalized surface.

2. Experimental

The plasma treatment experiments were conducted in a glass vacuum chamber, which is pumped down to a base pressure of 2.5 $x10^{-2}$ mbar (Figure 1). The chamber is provided with two

parallel aluminum electrodes, separated by 6 cm distance, where the upper one is RF active and the lower is grounded, serving as substrate holder as well. pNIPAAm layers were deposited on silicon substrates (IR transparent) by spin coating of 5% solution in chloroform. The plasma treatment of pNIPAAm was carried out at 20 W RF power (13.56 MHz, capacitively coupled) by feeding the reactor with 30 sccm of argon which established a working pressure of 0.51 mbar. The treatment time was set in the range from 30 seconds to 10 minutes.

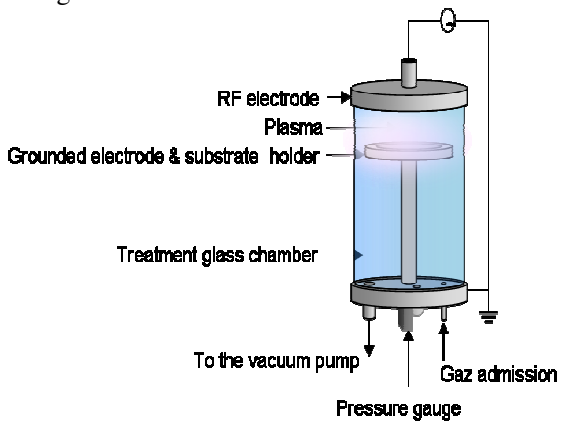


Figure 1. Experimental set-up used for plasma treatment of pNIPAAm thin films

The surface topography of pNIPAAm thin films was investigated by Atomic Force Microscopy (AFM), using a Park Systems apparatus, working in non-contact mode, for various areas ranging from 10x10 μm^2 down to 2x2 μm^2.

The chemical structure of the argon plasma treated PNIPAAM coatings was determined by Fourier Transform Infrared spectroscopy (FTIR) measurements performed with a JASCO 6300 spectrometer in the range 400-4000 cm^{-1}, with a resolution of 4 cm^{-1}.

The cell adhesion and detachment experiments were performed using fibroblast line L929 cells. A volume of 2 ml of cells suspension in proper medium containing phenol red, 10 % FCS (Fetal Calf Serum) and 0.1 % penicillin/streptomycin were dropped onto the sterilized samples surface, and kept in a CO_2 incubator at a temperature of 37 ºC. Prior to seeding on the samples, the cells were detached with trypsin, re-suspended in medium and afterwards brought to the desired concentration of 2×10^6 cells/ml. A volume of 15 µl of this suspension was uniformly placed onto the samples surface and 1.5 ml of medium was added in each well. The biological system was incubated at 37 °C, 5% CO_2 in humid atmosphere. Incubation time was set to 48 hours for viability cells testing, the cell detachment study and cell morphology analysis.

The optical observations of the samples submitted to biological tests have been performed at magnifications up to 40x using an inverted Olympus microscope (CKX31), equipped with a CCD camera.

3. Results and discussion

3.1. Material characterization

AFM investigations on untreated and plasma treated pNIPAAm surfaces, presented in Figure 2, reveal that RF argon plasma treatments induced a clear modification of the material surface regarding the topography. The untreated pNIPAAm coatings present a smooth surface with a roughness RMS of

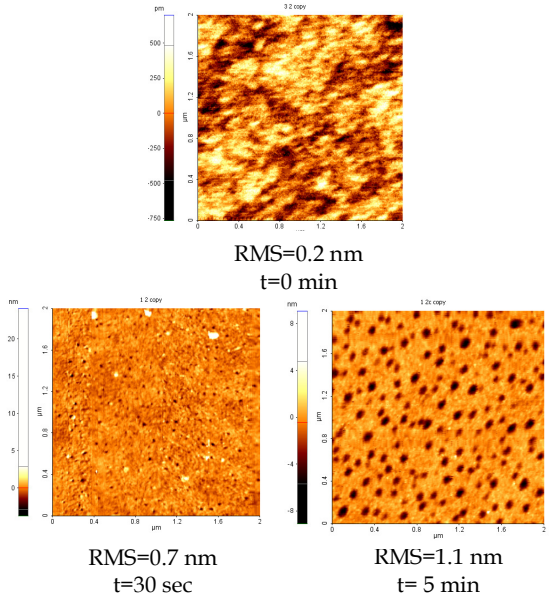


Figure 2. AFM images of the pNIPAAm coatings for various argon plasma treatment times

only 0.2 nm, without cracks or pores. Instead, in the case of plasma treated layers, the appearance of small pores on the pNIPAAm surface is obvious, even at short treatment time of 30 s. This is due to the etching effect induced by Ar ions present in the discharge. Intermediate treatment times, in the range of 1-5 minutes, lead to an increase of pores size up to ~75 nm, accompanied by only a slight effect on the surface roughness RMS.

FTIR technique allowed the investigation of the chemical bonds of the pNIPAAm coatings treated by RF argon plasma for various exposure times. Typical FTIR spectra of pNIPAAm materials, presented in Figure 3, reveal the presence of specific bonds corresponding to secondary amide C=O

stretching of amide I bond at 1646 cm^{-1} and respectively to N-H bending of amide II bond at 1540 cm^{-1}. Additionally, in the high wavenumber region, one can notice the presence of secondary amide N-H stretching around 3308 cm^{-1} and the band at 3435 cm^{-1} associated to free N-H stretching [11]. The deformation bands of isopropyl methyl radical (-$CH(CH_3)_2$)) are present at 1367 cm^{-1} and 1388 cm^{-1} [10, 12]. Specific vibrations of CH_2 and CH_3 radicals in the polymeric thin films are evidenced through the superposition of vibrations of CH_2 scissoring and CH_3 rocking around 1461 cm^{-1}, as well as the symmetric and asymmetric stretching modes of CH_3 at 2874 cm^{-1} and 2970 cm^{-1}, respectively, and to the asymmetric stretching vibration of CH_2 at 2934 cm^{-1} [13]. Upon plasma treatment, the intensity of all the above mentioned peaks is decreasing due to the etching effect which diminishes the amount of material present on the substrate that vibrates in the infrared region. At the same time, the absorption band associated to the COOH vibration situated around 1720 cm^{-1} is increasing in intensity. Its presence suggests an enhanced wettability induced by the presence of the polar groups which most probably appears upon exposure of treated samples to the open atmosphere.

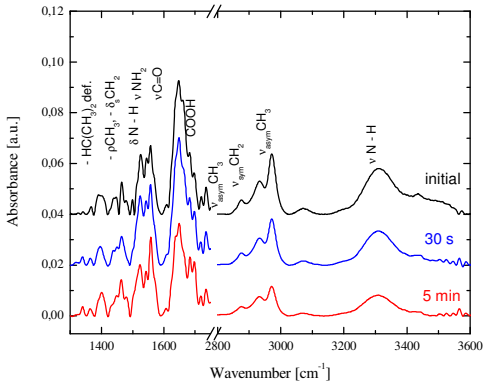
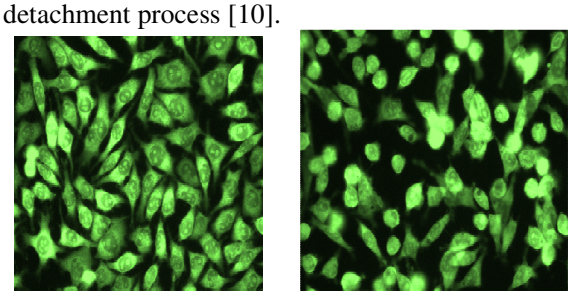


Figure 3. FTIR spectra of untreated and argon plasma treated pNIPAAm material

3.2. In vitro biological investigations

The fluorescence micrographs reveal that the L929 fibroblast cells proliferation strongly depend on the plasma treatment time (Figure 4), these cells presenting flat and spread morphology, typical for materials with high biocompatibility, only for low treatment time (30 seconds). At higher treatment time, namely at 5 min, the cellular morphology is clearly modified, with several cells presenting rounded shape. Furthermore, we can observe that the number of adhered cells onto pNIPAAm surface also depend on the plasma treatment time, being lower for longer treatment time. Such behavior is explained by the high surface porosity of the surface at long treatment time which inhibit the formation of cells monolayer interfering in the attachment detachment process [10].



a) 30 s b) 5 min

Figure 4. Images of the L929 fibroblast cells grown on the argon plasma treated pNIPAAm surface.

"Cell Titer 96Aquous One Solution Cell Proliferation Assay" Promega kit was used for the viability and proliferation testing of the cells. This kit uses the mitochondrial reduction of a tetrazolium salt (MTS) into a formazan product as metabolic marker, soluble in the culture medium. Such reaction is induced only by the viable cells. The 48 h incubated samples were placed in fresh wells containing 400 µl of viability solution (20% MTS in MEM solution). The samples were incubated for 3 h at 37°C, 5% CO_2 in humid atmosphere, and the resulted MTS solution was transferred into a 96 well plate. A TECAN Sunrise Basic plate-reader was used for determining the optical absorption at 490 nm, typical for this assay.

For all plasma treated samples, the viability was higher than that of the spin-coated pNIPAAm initial sample, of 76%. The highest viability was obtained for the shortest treatment time of 30 s, reaching a value of 105% in respect to the witness. This fact suggest that even mild conditions are efficient for improving material biocompatibility. On the other hand, for higher plasma treatment times the viability is below 90%, most probably due to the development of surface porosity.

The cell detachment experiments were performed by monitoring the number of cells on the surface while the temperature was decreasing in time from 37°C to 20°C (ambient temperature). In Figure 5 we present a sequence of 3 images of a pNIPAAM sample treated in plasma for 30 s and incubated with cells, after 0, 20 and 30 minutes upon the cooling procedure started.

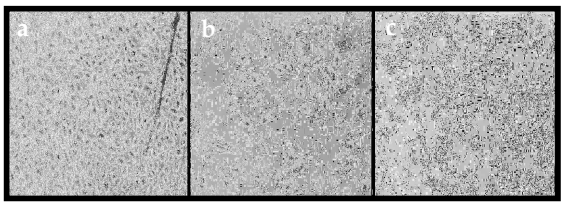


Figure 5. Cell detachment sequence images of pNIPAAm thin films treated for 30 s in argon plasma, upon cooling, after: a) 0 min; b) 20 min; c) 30 min

One can see that for the 30 s treated pNIPAAm thin films under normal culture temperature (Figure 5a) the cells were well attached to the surface and were proliferating. When the pNIPAAm growth support was cooled down, the cells are less spread and rounder, as shown in the Figure 5b). The detachment phenomenon is induced by the temperature decrease due to the hydration of pNIPAAm chains that present the LCST point around 32 °C. This is clear at longer cooling times, presented in Figure 5c), in which most of the cells are rounded and started to float off the substrate. By counting the number of cells on the pNIPAAm surface as function of cooling time (equivalent to a temperature decrease of ~0.4 degrees/min), we could evidence a decrease of cell number higher than 90% after 45 minutes, with almost linear temporal behavior leading to a detachment rate of ~6cells/min.

4. Conclusions

Plasma treatment in argon atmosphere was utilized for the functionalization of pNIPAAm thin films obtained by spin coating. The Ar ions formed in the plasma conducted to polymer etching and formation of porous materials, with pore dimension and density increasing by the Ar plasma treatment time. FTIR measurements evidenced the presence of pNIPAAm specific bonds and the formation of COOH bonds upon plasma, increasing the material wettability.

At T=37°C (above the LCST) cells adhere and proliferate onto the pNIPAAm dehydrated surface with viability increased with more than 25 % as compared to the control surfaces (not treated).

At a sample temperature of 20°C (below the LCST) pNIPAAm film rehydration occurs resulting in almost complete cell detachment.

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