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Effect of synthesis variables of plasma synthesized polymers on growth of HepG2 cells

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Abstract: Low pressure plasma polymer films were synthesized using pyrrole and allylamine monomers and adding iodine was used (or not) for the reaction in both cases. They were polymerized on glass substrates under the same reaction conditions. Polymerization of allylamine was also studied at different operating powers. These thin polymer films were used as culture surfaces for HepG2 cells, a cell line derived from a human hepatoma. The proliferation, differentiation and two-dimensional propagation until obtaining monolayer of the cells was studied on the different synthetized films and correlations were established between the conditions of synthesis, the physicochemical characteristics obtained and the performance as substrates for the cellular growth.

The deposition of plasma polymer films has potential applications in the modification of surfaces such as microelectronics, solar cells, gaskets, corrosion protection and biomaterials (Tserepi *et al.,* 2016, Yanling *et al.,* 2014). Plasma deposited polymer layers can improve the surface characteristics of the materials and generate biocompatible coatings. Plasma treatment is reproducible, clean, does not generate toxic waste, does not require complicated separation processes and allows modification of materials without altering its bulk properties. Plasma can be used to modify a surface by implantation, abrasion, eroding, or reservoir of reactive species.

Plasma modified surfaces from different monomers including those having amino groups such as pyrrole, allylamine, propylamine and cyclopropylamine have been used as substrates to study the interaction of cells with materials (Tserepi *et al.*, 2016, Boespflug *et al.*, 2016, Manakhov *et al.*, 2016, Klages *et al.*, 2013). Sterile, plasma-treated samples can be used directly in live animals or for cell culture, and they are stable even after several cycles of sterilization and different substrates can be coated (Maslakcia *et al.*, 2017, Hawker *et al.*, 2015, Cheng *et al.*, 2013). It may be interesting to refer that Zhang (2005) and Chen *et al.*, (2004) have studied DNA hybridization on plasma-polymerized allylamine films.

Our laboratory has been working in the synthesis and physicochemical characterization of polymer films synthesized from different monomers with plasma (Morales *et al.*, 2002, Cruz *et al.*, 1999 and 1997). It has been reported that the plasma synthesized pyrrole polymers are integrated into the surrounding tissue when used as implants in traumatic lesions of spinal cord, and they function as neuroprotective agents, resulting a degree of functional recovery after injury that was similar to the non-implanted controls (Alvarez-Mejia *et al.*, 2015; Olayo *et al.*, 2012). HepG2 cells are a cell line derived from a well-differentiated human hepatoma, and they respond to injury by rapidly turning off differentiation and proliferation, and Wang *et al.*, (2006) have studied the behavior of hepatocytes on hydrophilic surfaces.

This work presents an *in vitro* comparative study of growth of HepG2 cells, on plasma-polymerized thin polymer films, polypyrrole (PPPy) and polyallylamine (PPAa). Also, the same polymers were studied after the addition of iodine during the reaction (PPPy-I and PPAa-I), and in PPAa films synthesized at different reaction powers.

For that purpose, glass substrates were coated with different polymers. The plasma polymerization reactor consisted of a flanged tubular Pyrex glass reactor with stainless steel electrodes, assembled in a similar configuration

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to the one described elsewhere (Morales *et al.*, 2002, Cruz *et al.*, 1999 and 1997). The diameter and length of the reactor were 9 cm and 25 cm respectively. Glass substrates were used inside the reactor and a plasma discharge at 100 W was used for 10 min to clean the surface of the glasses before a continuous monomer vapor was fed without carrier gas. Different films were prepared using 10 min of reaction by varying the power and the fed monomer. Pyrrole (Aldrich, 99%) and allylamine (Aldrich, 99%) were used separately and in some films iodine (Aldrich, 99.8%) was further fed. At the end of treatment, the reactor was shut down and kept closed for 30 min before the samples were recovered. One part of each batch was used for physico-chemical analyses and the other was used as a substrate for the hepatocytes culture.

To characterize the surface of coatings, angle measurements were made by a front photograph of the substrate, under a drop of distilled water. Angles were measured with the ImageJ® program. Six readings were made at different points on each sample and were averaged to yield a contact angle value. Structural analysis of the samples was done by infrared spectrometry of attenuated total reflectance, IR-ATR (Perkin-Elmer 2000).

HepG2 cells for cultures were obtained from ATCC, Rockville, MD, USA, and were cultured as published elsewhere (Gómez-Quiroz *et al.*, 2003). Known and equal cell concentrations were seeded onto standard polystyrene cellular culture dishes (Corning, 430165) as control and thin polymer films deposited on glass by low pressure plasma. The pre-seed count was performed using a Neubauer chamber, the initial cell density was 2×10^5 cells/mL. All samples were incubated in a humid environment with 5%CO_2 and 95%air; Williams medium (Sigma, EW4125) supplemented with 10% fetal bovine serum (Hyclone Laboratories Inc., Logan Utah, USA), penicillin (100 units/mL) and streptomycin (100 mg/mL) were used for culture. Samples were washed with a phosphate buffer-saline solution (PBS, Sigma_Aldrich, D 5773) every 24 h. The evolution of cultures was observed and recorded every 24 h, by means of a digital image capture system, coupled to a phase contrast microscope (Zeiss, Axiovert 25). For scanning electron microscopy, the seeded cell samples were prewashed with PBS and fixed with a 3:1 solution of methanol and acetic acid and using a HRSEM Jeol JSM-7600F scanning electron microscope.

Because a preceding study (Cruz *et al.*, 1999) has shown that pyrrole polymerization by plasma shows a direct relation between thickness of the deposited coating and the time of synthesis, we first studied the conditions for the synthesis of thin films, using PPPy, PPPy-I, PPAa or PPAa-I on glass coverslips, under conditions of 10 minutes of synthesis time, 25W and pressure of 1.6×10^{-2} Torr. The pyrrole polymer films showed a contact angle of 49±1° and those of allylamine 27±1°, while iodine did not influence the contact angle.

Afterwards, the operating power of the reactor was varied and only allylamine was fed, the reaction time was 10 minutes on glass coverslips, and the source power varied between 10 and 150 W. It was clear that higher contact angle values were associated to powers greater than 30 W (Table S1).

The IR spectra of the samples showed wide and complex absorption bands characteristic of plasma synthesized materials. Fig. S1 shows the ATR-IR spectrum of PPPy and PPPy-I. A strong presence of amines can be identified around the 3400 cm^{-1} band, then the signal at 2900 cm^{-1} is detected, suggesting the presence of C-H aliphatic groups. At 2100 cm^{-1} the vibration of the carbon-nitrogen triple bond is detected, these last two peaks indicate the breakage of pyrrole rings due to the energy of the particles in the plasma. The signal at 1630 cm^{-1} is attributed to flexion in amines groups. The signal in 750 cm^{-1} corresponds to C-H vibration.

Fig. S2 shows the IR-ATR spectrum of the allylamine polymer films, PPAa, and of allylamine with iodine, PPAa-I. The broad signals characteristic of plasma synthesized materials are also shown. Signals in the region of 3350 cm^{-1} and 1630 cm^{-1} can be distinguished and assigned to the presence of amines. Fig. S3 shows the spectra of the films obtained from allylamine at different reaction powers, shows the presence of amines in 3350 cm^{-1}, that was clearer at a power of 30 W, of 100 W or 150 W. This implies that increasing the power to more than 30 W fractures the monomer and part of the nitrogen is lost.

Then, HepG2 cells were seeded at a concentration of 2×10^5 cells/mL, on the PPPy_10, PPPy-I, PPAa and PPAa-I films; and all samples were cultured under the same conditions.

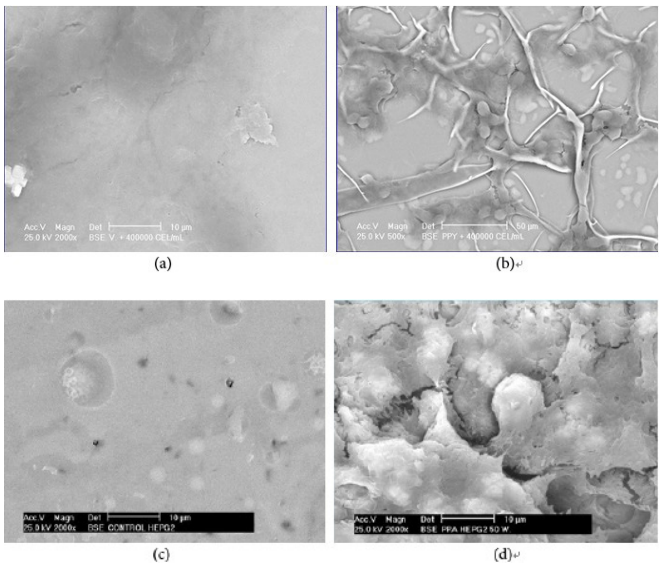

Figure 1. Scanning electron microscopy micrographs of the samples of PPPy withouth cells (1a), and HepG2 cells (1b), and PPAa without cells (1c), and with HepG2 cells (1d).

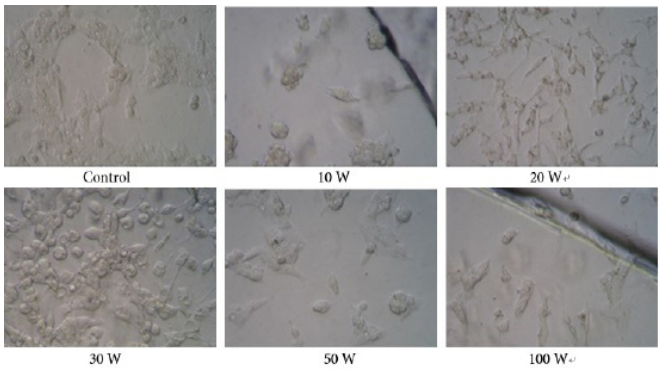

Figure 2. HepG2 cell growth in a culture dish (control) and on PPAa films synthesized at different powers.

Fig. 1 shows scanning electron micrographs of the samples of PPPy_10 without cells (1a) and HepG2 cells (1b) and PPAa without cells (1c) and with HepG2 cells (1d), morphology was somewhat different since the PPAa shows certain defects on the surface. However, both surface types showed cell growth on them.

Fig. 2 shows the cell growth of HepG2 cells on PPAa films synthesized at different powers, it is verified that there is a direct relationship between the polymerization power and cellular growth, the highest growth occurs on films synthesized at 30 W and it decreases as power increases above this value.

We have shown here that pyrrole and allylamine films synthesized by plasma on glass, were not toxic to HepG2 cells, allowed adhesion and enhanced cell proliferation. The addition of iodine did not modify the results. *In vitro* differences in adhesion, morphology and proliferation of cells seeded on treated and untreated carriers by plasma were observed. Cell differentiation was considered as an indicator of functionality by observing that cells adhere, grow and differentiate earlier on the polymers than on controls and they proliferate more rapidly.

Plasma synthesized PPPy, PPPy-I, PPAa and PPAa-I thin films show no toxicity and increase the adhesion and proliferation of HepG2 cells. In the case of PPAa, the synthesis conditions that increase the presence of amines (30 W) were related to a better performance of the substrates.

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