STUDY OF BIOACTIVE FILMS USING NATURAL MACROMOLECULES

Mariselma Ferreira[1], Valtencir Zucolotto[2], Marcelo Mulato[1],
Osvaldo N. Oliveira Jr[2]

[1]Departamento de Física e Matemática, FFCLRP-USP, Av. Bandeirantes 3900, Ribeirão Preto-SP, 14040-901, Brazil, mselma@ffclrp.usp.br
[2]Instituto de Física de São Carlos – USP São Carlos, CP 369, 13560-970, São Carlos-SP, Brazil

ABSTRACT

Thin polymer films produced by alternating bilayers of natural macromolecules, such as humic acid (HA) and natural rubber latex (NR) extracted from *Hevea brasiliensis*, were investigated for their cell-attachment interaction with human fibroblast cells. Layer-by-layer (LbL) films with up to 10 bilayers of PAH/HA or PAH/(HA+Latex) were deposited directly on tissue culture polystyrene substrates (TCPS). In control experiments, cast films were fabricated on TCPS using 0.5% (v/v) of fresh latex and 0.5 g/L solutions of HA in different proportions (latex:HA = 10:1, 5:1, 1:1, 1:2). Both PAH/HA and PAH/(HA+Latex) LbL films exhibited good performance on cell adhesion and growth. On the other hand, the cast films were found to be cell resistant and bioinert coatings. The viability for creating cell interactive materials has been demonstrated for these natural macromolecules using the LbL technique.

Keywords: Natural macromolecules, layer-by-layer, cell attachment, human fibroblast

1 INTRODUCTION

The control of interactions between living cells and modified surfaces is essential for tissue engineering and may be exploited in the fabrication of bioactive coatings. One possible method to produce such coatings is the layer-by-layer (LbL) technique, based on the spontaneous adsorption of oppositely charged materials onto a substrate, which has been used in various applications [1-3]. For biomolecules, in particular, Lvov and co-workers immobilized proteins in LBL films that remained active for long periods of time [4, 5]. The LBL technique has also been employed with synthetic polymers to engineer biomaterial surfaces [4-10] such as alternating layers of poly(allylamine hydrochloride) (PAH) and poly(styrenesulfonate) (PSS). The latter were used as substrate for immunoglobulin (IgG) immobilization and fabrication of a precursor film for immunosensing. Because of the possible preservation of bioactivity, LbL films have been largely used in biosensors [5], and recently for bioactive films containing growth factors [6]. Growth factors are water-soluble polypeptides involved in tissue regeneration and cell proliferation. Fibroblast growth factor (FGF) is one of these polypeptides participating in the cell mitosis of the fibroblasts, and analogously to other growth factors, has a short half-life. In ref. [6], LbL films were produced with the acid FGF/heparin complex as negative polyelectrolyte while poly(ethyleneimine) (PEI) was the positively charged polymer. The films produced permitted rapid proliferation of the cell fibroblasts as a result of the preservation of the growth factor bioactivity [6]. In addition to the successful incorporation of the proteins into a polymer network, the LbL technique was used to introduce cell adhesive proteins such as gelatin, collagen and laminin on polystyrene surfaces [7]. The latter study reported that mouse embryonic cells grew best on surfaces coated with gelatin and collagen. Cell adhesion is a critical process for biomaterials, being associated with the cell proliferation and development [8]. Biological surfaces designed by manipulating the pH and ionic strength conditions of the alternating multilayers provided cell attachment and proliferation control leading to micropatterned film fabrication [9-10].

Here we investigated the interaction of fibroblasts cells on bio-surfaces fabricated with humic acid and natural rubber latex. Humic acids (HAs) are organic macromolecules bearing colloidal and polyelectrolytic characteristics, which result from the microbiological decomposition of animals and vegetables. They can be found in waters, soils, vermicomposts and peats. Vermicomposts stem from microbial degradation in the intestine of earthworms whereas peats are dark color organic matter with varied decomposition degrees, usually found near rivers and lakes [11]. HAs have already been used in LbL films as highly sensitive pesticide sensors [11]. Natural rubber (NR) latex, a polydispersive system extracted from the *Hevea brasiliensis*, comprises 40-45% of 1,4 poly-cis-isoprene, 4-5% of non-rubber constituents such as proteins, lipids and 50% of water. NR has been used in bandages (Biocure®) and is a strong candidate for biomaterials [12, 13]. Proteins extracted from NR latex have been studied using the choriollantoic membranes model, with the results pointing to a strong angiogenic activity [14]. With the LbL films produced in this study we demonstrate the viability of creating cell interactive materials from natural macromolecules. Even the films produced with HA (a residue from organic matter

decomposition) showed good biocompatibility. On the other hand, cast films produced with the same materials were found to be bioinert, which demonstrated the importance of the deposition method and film architecture for biocompatibility.

2 MATERIALS AND METHODS

2.1 Materials

The natural rubber latex extracted from a RRIM 600 clone of *Hevea brasiliensis* was preserved with ammonium solution to avoid coagulation and to keep the latex stability. PAH, MW = 65,000 $g.mol^{-1}$, was purchased from Aldrich Co. and used without purification. HA from peat was obtained from a tropical region in São Paulo, Brazil. Details concerning the HA extraction, purification and characterization can be found in ref. 15. Briefly, HA was extracted from peat using NaOH solution and purified according to the International Humic Substances Society (IHSS) [16].

2.2 Preparation of LbL films

We produced LbL films with up to 10 bilayers of PAH/HA or PAH/(HA+Latex) deposited directly on tissue culture polystyrene substrates (TCPS). The PAH solution was used at a concentration of 0.5 $g.L^{-1}$ and pH 6.0. For HA, a concentration of 0.5 $g.L^{-1}$ and pH = 9.0 was set in order to ensure complete dissolution of HA. At this pH most COOH groups are ionized, and ionized phenolic groups can also participate in the adsorption process [11]. The sequential deposition of multilayers was carried out by immersing the substrates alternately into the polycationic PAH solution for 3 min and in the anionic HA or (HA+latex mixture 50% v/v) solution for 5 min. After each layer deposition, the substrate/film system was rinsed in the washing solution and dried under a N_2 flow. The same procedure was repeated to deposit 20 bilayers of the PAH/HA or PAH/(HA+Latex) onto a quartz substrate and the growth of the multilayers was monitored using UV-VIS spectroscopy. In control experiments, we obtained cast films on TCPS substrates using 0.5% v/v fresh latex solution and 0.5 g/L solutions of HA in different proportions (latex:HA = 10:1, 5:1, 1:1, 1:2). The solutions were cast onto the substrate, which was dried in an oven under vacuum.

2.3 Human fibroblast culture

Human fibroblast cells were cultured on the TCPS substrates using DMEM (Dulbbeco's Modified Medium) and HAM-F10 essential medium containing 10% SBF (serum bovine fetal). In addition, the medium was supplemented with 1.0 % (v/v) of streptomycin (10mg/ml) and penicillin (10.000 U/ml) purchased from GIBCO, 1.5% (v/v) ciprofloxacin (Bayer) and Kanamicyn (Amresco). The fibroblasts were then seeded onto the sterilized multilayers at the density of approximately 10.000 cell/cm^2 in serum-containing media. An optical microscope (10x magnification) was used to take images 1, 2 and 4 days after cell incubation.

3 RESULTS AND DISCUSSION

The absorption spectra displayed in Figure 1 indicate a broad band peaking at 214 nm for the HA+latex mixture, which is blue-shifted with a maximum at 195 nm for the PAH/HA+latex 20-bilayer LbL film. The latter indicates a H-type aggregation in the film. For the PAH/HA system, there was no difference in the absorption spectra between solution and LbL film (results not shown).

Figures 2 and 3 show the growth of PAH/HA and PAH/HA+Latex multilayers monitored with UV-VIS spectroscopy. The electronic absorbance of the films /HA increased linearly with the number of bilayers, suggesting that the same amount of material was adsorbed at each deposition step (see Fig. 4).

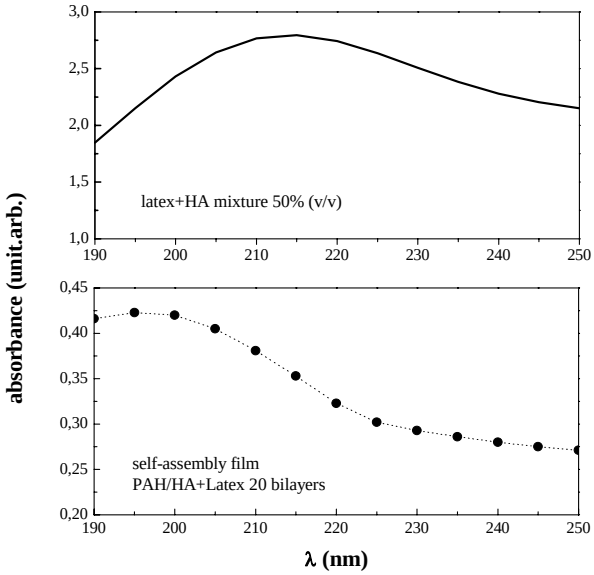

Figure 1: Electronic absorption for the HA+latex systems.

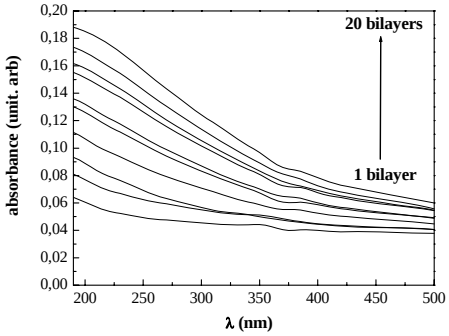

Figure 2: Electronic absorption for PAH/HA LbL films containing different numbers of bilayers.

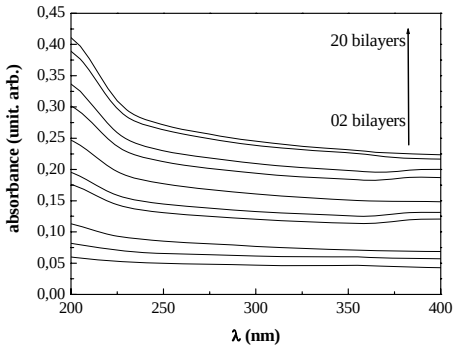

Figure 3: Electronic absorption for PAH/HA+latex LbL films containing different numbers of bilayers.

Comparing the slopes of the linear growth for the two systems studied here, Figure 4, one notes adsorption in the PAH/HA+ latex films was more efficient, which points to the latex adhesion properties facilitating film formation.

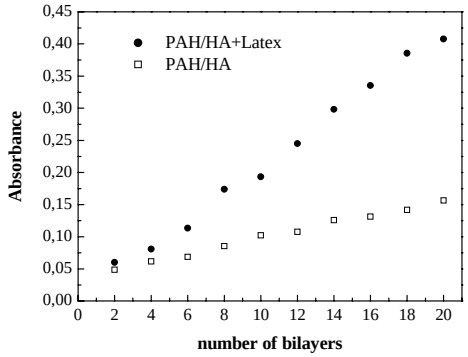

Figure 4: Linear dependence of the absorbance at 250 nm as a function of number of bilayers for PAH/HA and PAH/HA+Latex LbL films.

The PAH/HA and PAH/(HA+Latex) LBL films exhibited good performance on cell adhesion and proliferation, as illustrated in Figures 5a and b. The images correspond to contrast micrographs of human fibroblasts cells acquired after 4 days of seeding. The fibroblasts show substantial attachment, spreading and typical morphology for these cells. On the other hand, the cast films from fresh latex:HA mixture were cell resistant surfaces and bioinert coatings, as indicated in Figures 5c and d.

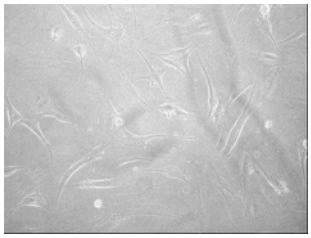

Figure: 5a. Cells grown on PAH/HA multilayer film

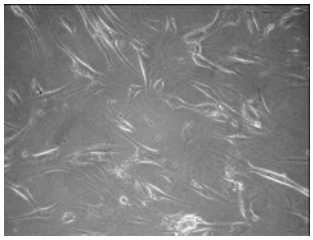

Figure: 5b. Cells grown on PAH/(HA+Latex) multilayer film

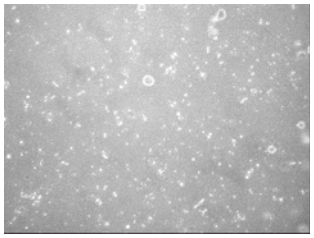

Figure: 5c. Cells on cast film of latex:HA =10:1

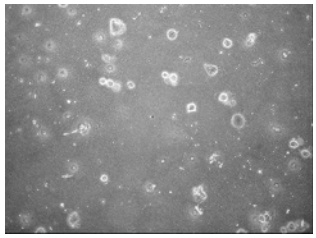

Figure: 5d. Cells on cast film of latex:HA=1:1

4 CONCLUSIONS

Natural macromolecules such as humic acid and natural rubber latex were investigated as potential substances to fabricate bioactive surfaces. Nanostructured films were produced using the LbL technique, with cast films of the same components being employed in control experiments. Cell attachment performance of the films was evaluated by seeding fibroblasts cells on the surfaces and a good response was obtained in the nanostructured films. Cast films were proven cell-resistant surfaces, with no cell spreading or proliferation. In conclusion, we demonstrated a simple method to produce a cell interactive material from natural macromolecules using the LbL technique even for the film produced using humic acid considered as an organic matter residue.

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