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adriana.delmonaco@usp.br

Grafting of Hyaluronic Acid to PLDLA, used as coating of the models of PLLA bioresorbable coronary stents.

Del Monaco ADM*§, Duek EAR#, Andrade AJP§ and Malmonge SMM*

**Federal University of ABC (UFABC), Brazil. § Dante Pazzanese Institute of Cardiology - University of São Paulo (IDPC - USP), Brazil. # Pontifical Catholic University of São Paulo (PUC-SP), Brazil.*

Abstract. Coronary artery disease has been leading cause of death in the world, angioplasty stent implantation is an important strategy in these cases. Studies indicate that biodegradability, immobilization of antiproliferatives and bioactive molecules in stents, are the characteristics of future generations of these devices. Amongst them, hyaluronic acid contributes to decrease of the aggregation and proliferation of cells between artery layers and the implanted device. For this purpose, poly (L-lactic acid) (PLLA) bioresorbable coronary stents with hyaluronic acid (HA) grafting in poly (LD- lactic acid) (PLDLA) were developed. The models were characterized as their thermal properties with Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetric Analysis (DSC), Fourier Transform Infrared Spectrometry (FTIR), Wettability, with study of Contact Angle using Water swelling. PLDLA ADH-modified HA grafting presented more hydrophilic surface characteristics, ideal for coating material of this devices. In conclusion, grafting of hyaluronic acid in the PLDLA matrices confirmed, by technics used at this project. The coating of models of PLLA with PLDLA-HAADH made by dip coating technic.

Keywords: *Polimeric biomaterials, Bioresorbable stents, PLDLA, PLGA, PLLA, HA.*

Introduction. Coronary artery disease (CAD) remains the leading cause of death in the world, and Brazil is no different, accounting for 29.4% of deaths per year across the country. These data places Brazil among the 10 with the highest mortality rate due to CAD. [1, 2, 3, 4, 5]. Angioplasty is a procedure performed minimally invasively, as a treatment indicated for coronary arteries with stenosis. The access performed via catheterization, where inflation of a balloon, inside the atheroma plaque, against the walls of the vessel, unclogging the injured artery and restoring blood flow. This technique based on possibility of regression of an atherosclerotic narrowing, which was occasionally observed by Dotter in the 60's, where restoration of the flow of an iliac artery occurred through the passage of a diagnostic catheter. This observed phenomenon gave rise to the concept of vascular dilation for treatment of stenosis [6, 7]. This treatment is related to cases of recurrent restenosis, a process similar to that of stenosis, which occurs in such a way that platelet aggregation, cell proliferation and negative remodeling of artery eventually obstruct the passage of blood through the vase. For years, restenosis has been one of the major challenges to be overcome in coronary translumial angioplasty. Within this scenario, platelet aggregation can be

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treated clinically with antiplatelet medications such as Acetylsalicylic Acid (AAS - Bayer ®) and Clopidogrel Bisulfate (Sanofi - Aventis ®). However, negative remodeling problem could be solved mechanically, with use of stents [8, 9, 10]. One of the first experiments was implantation of metallic stents that were expandable by Palmaz et al. in 1984, who used stainless steel devices contained by a membrane, removed after the positioning of it inside of atheroma plaque, before expansion with balloon. This procedure was a very important step in the treatment of coronary diseases. The first coronary stent implantation in Brazil was performed in 1987 at the Dante Pazzanese Institute of Cardiology (IDPC), by Dr. José Eduardo Sousa and team [11, 12, 13].

The first English bioresorbable vascular scaffold (BVS) were developed by Tamai et al. in the early 2000s. They were made from biocompatible and bioreabsorbable polymer materials. The main polymers used in the development of bioresorbable stents are the same ones used in the coating of drug-eluting stents, namely poly (-L-lactic acid) and poly (-L-lactic acid -co-glycolic acid) (PLLA and PLGA). Bioresorbable stents are temporary devices that provide transient mechanical support, in addition to the incorporation of antiproliferatives, such as the second generation drug-eluting stents (DES), being totally degraded and absorbed by the body within 2-3 years average. The same period indicated by Nordman in 2006, regarding the period of higher mortality for patients with drug-eluting stents [14, 15, 16].

The review carried out by Mani et al. Points out several foci to be sought in development of stents: hemocompatibility, hydrophobicity, anti-inflammatory properties, surface conformability, easy sterilization, immobilization of drugs and, finally, point to biodegradability as one of the main properties of these devices future generations. The first commercially available bioresorbable stents were fabricated by Abbot® laboratory in 2011 and the first implant procedures of these devices were performed by Dr. Alexandre Abizaid's team, also at the Dante Pazzanese Institute of Cardiology [9, 11, 15, 17, 18, 19].

The extracellular matrix (ECM) is a structure that emerged evolutionarily, from the multicellular organisms, with the function of promoting the structure of the tissues, conferring flexibility between the cells. Its main components are glycosaminoglycans (GAGS): proteins covalently bound to polysaccharide chains known as proteoglycans, fibrous proteins, such as collagen and elastin, and adhesive, such as fibronectin and laminin [20,21]. Due to its non-stick characteristics, HA is a suitable biomaterial to reduce cell adhesion in implanted devices. HA can be found in vascular tissues and prosthetic heart valves, with significant potential to produce biocompatible materials for cardiovascular tissues engineering. In addition, because it is charged negatively, it can be easily integrated in layer by layer, for the device surfaces coating [22]. Finally, because HA is an important component to be considered in the process of restoration of endothelium physiological conditions after angioplasty with stent implantation, HA coating has been presented as an interesting strategy for the purposes of present study.

Materials and methods. PLLA films and tubes, material for central structure of the model and films of PLDLA, coating material and, therefore, were used for hyaluronic acid grafting.

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Bioresorbable Polymers: Films of the PLLA (molar mass distribution rate ~ 140,000 Da - Laboratory of Biomaterials of the Pontifical Catholic University of São Paulo) and PLDLA (molar mass distribution rate 100,000-160,000 Da - Laboratory of Biomaterials of the Pontifical Catholic University of São Paulo) polymers were obtained using solvent evaporation method, 2.5 g of polymer was dissolved in 50 ml of chloroform under constant stirring and at room temperature for 1 hour. After complete dissolution, polymers were deposited in Petri dishes, previously silanized, to facilitate the subsequent removal of the formed film. The silanization of plate was carried out with silicone oil in an oven at 200 ° C for 2 hours, followed by cooling and removal of excess oil with surface washing with detergent. The plates containing solution were capped with exhaustion at room temperature and, after complete solvent evaporation, after 3 to 4 days average, films were removed from the plates and stored under vacuum at room temperature.

HAADH engrafting in PLDLA matrices (PLDLA-HAADH): The protocol used involved the chemical modification of HA with adipic dihydrazide (ADH), activation of PLDLA with N, N-dicyclohexyl carbodiimide (DCC) and N-hydroxysuccinimide (NHS) and subsequent grafting of HAADH on PLDLA and activated PLGA. In this way, to HA modification it was adopted previously reported procedure [22, 23]. HA was dissolved in distilled water to obtain a solution with a concentration of 5mg / mL. Then, molar excess (20-30 fold) of ADH, with a pH value adjusted to 4.8-5.0 was added. After approximately 2 h the reaction was quenched by adjusting the pH value to 7.0. The modified HA was purified by dialysis to obtain solid HA-ADH. 250mg of PLDLA was also dissolved separately in a mixture of 5mL of dimethylsulfoxide (DMSO), 3.1mg DCC and 1.73mg NHS. Another solution of HA-ADH in DMSO (5 mg / 5 mL) was prepared. After complete dissolution, the PLDLA solutions were mixed with 5 mL HA-ADH solution for reaction at 40 ° C for 24 hours under constant stirring. After reaction the products were dialysed in deionized water under stirring at room temperature for 2 days using dialysis membrane (cut off 14000 Da). The products were also resuspended in 2ml chloroform and, after homogenization, films were obtained by solvent evaporation.

Preparation of the models: PLLA tubes: PLLA in the form of tubes were used as the internal structure of stent prototype. The prototype consists of such layered structures of HA-grafted PLDLA. Samples of PLLA tubes were prepared by the dip coating method, ie deposition of polymer film on surface of guide metal cylinders of suitable diameter, in the order of 2 to 5 millimeters, approximating the dimensions of a coronary stent. The deposition of the film was carried out by dipping the cylinder in solution of polymer, followed by evaporation of solvent, even used in making of the films. The procedure was repeated the number of times necessary to obtain the desired thickness for tube, Fig 1.

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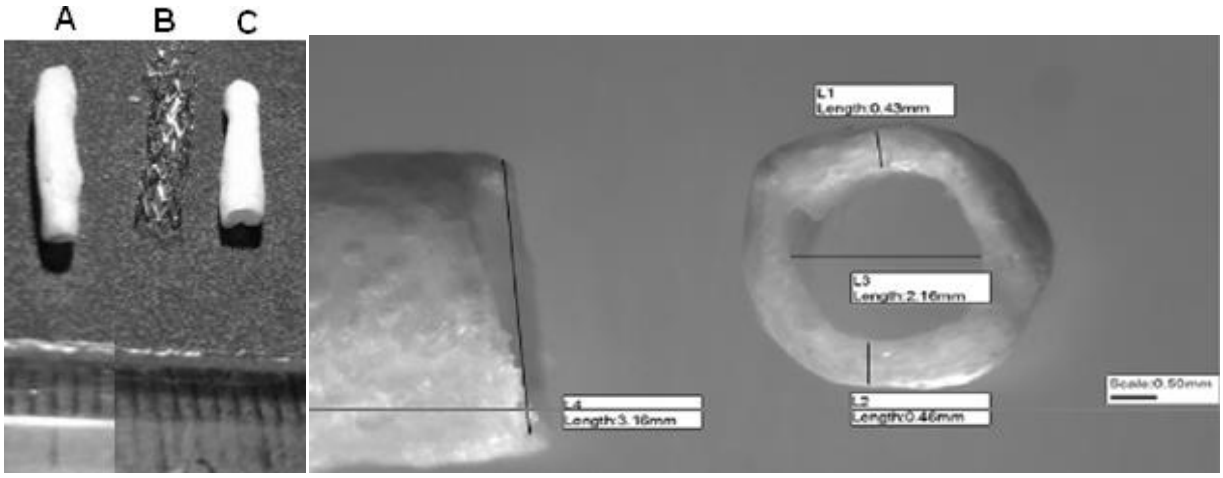

FIG. 1. Photographing PLLA tubes before and after coating compared to a commercial coronary stent (Cronus® - Scitech) A) PLLA coated with PLDLA; B) Cronus® metal stent; C) PLLA without coating; And Stereomicroscopy with uncoated PLLA tube measurements.

Coating of the models: PLLA tubes: After the preparation of inner layer of PLLA, PLDLA coating was continued for studies on choice of the best biomaterial to be used as a coating of prototype, in addition to the determination of coating methodology for fabrication and characterization of the prototype. The method used was also the dip coating as described in previous item, but only one immersion cycle was performed, depositing only one layer of PLDLA in the PLLA matrices.

Characterization of the models: Fourier Transform Infrared Spectrometry. The Fourier Transform Infrared Spectrometry (FTIR) (Variant-Agilent® 640-IR FT-IR), evaluating the profile of active vibrational spectra between 400 and 4000 cm¹, by transmittance and specular and diffuse reflectance.

Evaluation of thermal properties: Thermogravimetric analysis. The thermal properties were evaluated by thermogravimetric analysis (TGA) (TA Instruments®, TGA Q500), which was performed using an inert atmosphere with a flow of Nitrogen at a flow rate of 20 ml / min, with a scanning from ambient temperature to 600 ° C with a speed of 10 ° C per minute.

Evaluation of thermal properties: Differential scanning calorimetry. In thermal differential scanning calorimetry (DSC) analysis (TA Instruments®, Q-series), samples were submitted to the heating-cooling-heating cycle, with a heating rate of 10 ° C per minute and a cooling rate of 5 ° C per minute in an inert atmosphere of Nitrogen with flow Of 20ml / min.

Swelling: In addition, swelling in deionized water and analysis of water wettability by contact angle were evaluated. To evaluate swelling, three samples of each film (PLDLA with and without HAADH) were weighed and immersed in deionized water. Each sample was weighed again after

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15, 30, 45 and 60 minutes and after 24 hours in contact with deionized water. The averages and standard deviation were used to present these results, in percentage by mass of water in sample.

Wettability: The samples were evaluated for contact angle to characterize the wettability using a tensiometer (Attension® and Contact Angle® software). The equipment captured 20 images, one every second, and the contact angle determined for each image. The wettability was defined in terms of the mean obtained from left and right sides of the contact angles (θ), after stabilization, plus standard deviations.

Results and discussion.

FTIR: The results of the FTIR are shown in Fig. 2, below, spectra obtained for PLDLA samples were compared to the spectra of the samples with HAADH grafting and with the spectra of pure HA. It was possible to observe a small change in PLDLA-HAADH spectrum in the characteristic range of the water molecule. This may indicate the presence of water in sample due to the absorption of moisture from the air, by the rather hydrophilic character of HA. It was not possible to observe any other alteration in the spectrum of PLDLA with HA, one hypothesis is that the sensitivity of this method was not sufficient to detect small amounts of grafted HA.

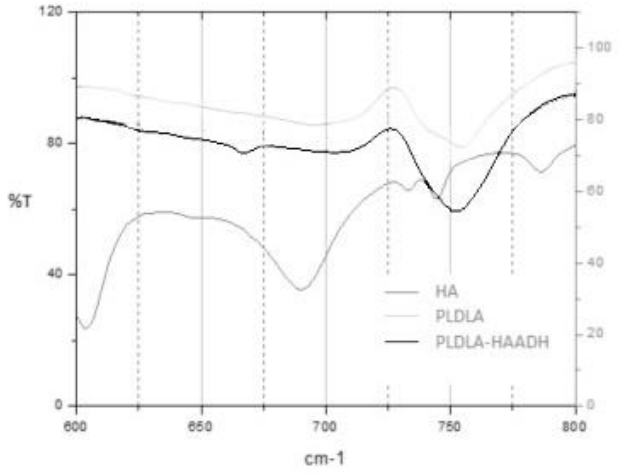

FIG. 2. Analyzes obtained by FTIR spectroscopy for the samples presented in the legend.

TGA: The thermal properties evaluated by thermogravimetric analysis (TGA) are presented in Fig. 3, which comprise the mass percentages as a function of temperature. Curves for PLDLA samples and HAADH grafting, respectively, can be observed. It is also possible to observe an alteration around 100 ºC, characteristic of presence of water, absorbed by HA.

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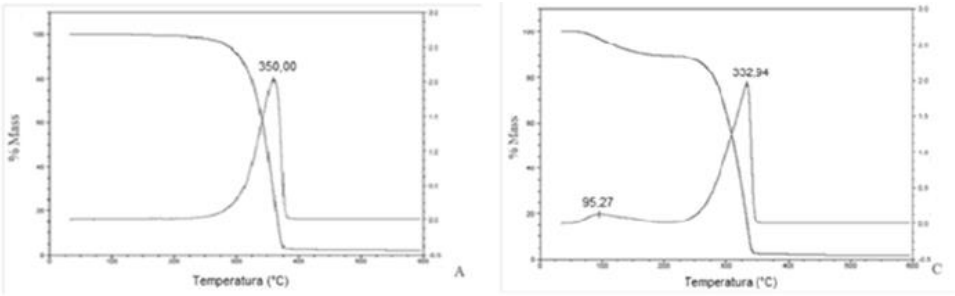

FIG. 3. Curves of TGA PLDLA control and PLDLA with HA and ADH.

DSC: Differential thermal analysis (DSC) presented results shown in the graphs of Fig. 4, PLDLA, before and after HAADH grafting. No changes could be found by this method.

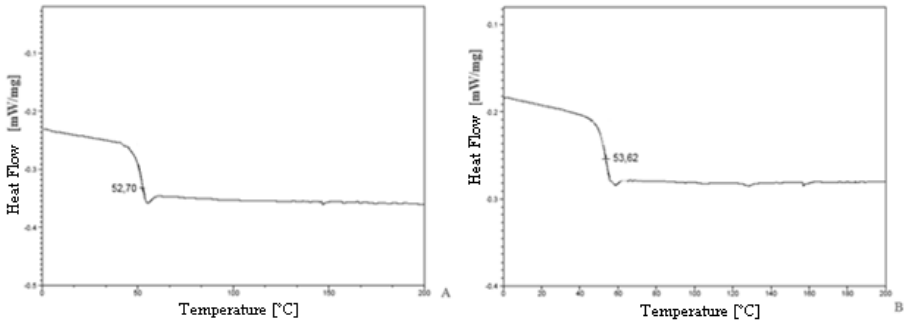

FIG. 4. DSC curves PLDLA, PLDLA with HA and ADH.

Swelling: The swelling values of polymers in deionized water are shown in Fig. 5, where it is possible to observe a greater increase of water absorption in the films grafted with HAADH. It is also possible to observe a change in transparency of the films after 1 day of swelling, according to Fig. 6, where the PLDLA-HAADH films presented a very marked whitish appearance, whereas this behavior was not evident in controls, which remained transparent as Before being tested.

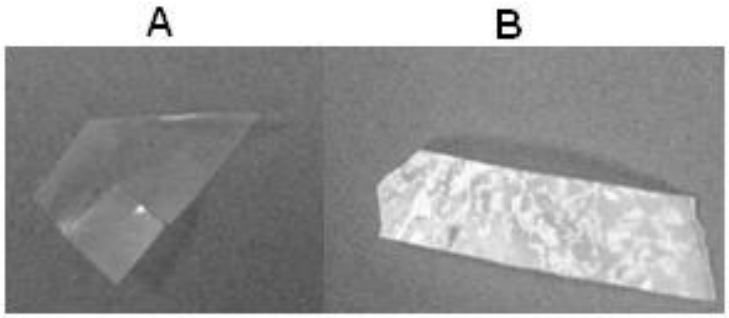

FIG. 5. Swelling: Photograph of the samples used in the test, in the time of 3 days. A) PLDLA control and B) PLDLA-HAADH.

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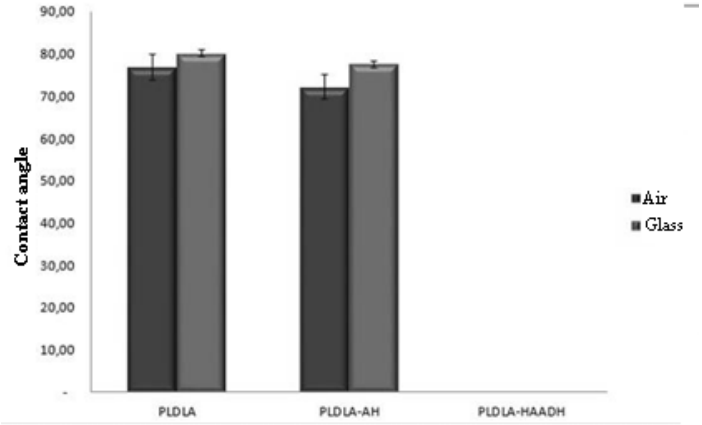

FIG. 6. Mean values of the contact angles between deionized water droplet and air and glass surfaces of the PLDLA and PLDLA-HAADHDH polymers.

Contact Angle: Measurements of contact angle between a liquid droplet and the surface of polymer allow the wettability of surface to be assessed to the liquid in question. In this study, deionized water was used, which allowed to evaluate affinity of surfaces by water, that is, the hydrophobicity of the material. The contact angle values between deionized water droplet and surface of polymers under study are shown in Fig 7, which comprise the averages, between left and right angles, taken after stabilization of droplet deposited on the surface, plus deviation Standard and analysis of variance to verify the existence of differences between the groups, for a confidence interval of $p < 0.05$

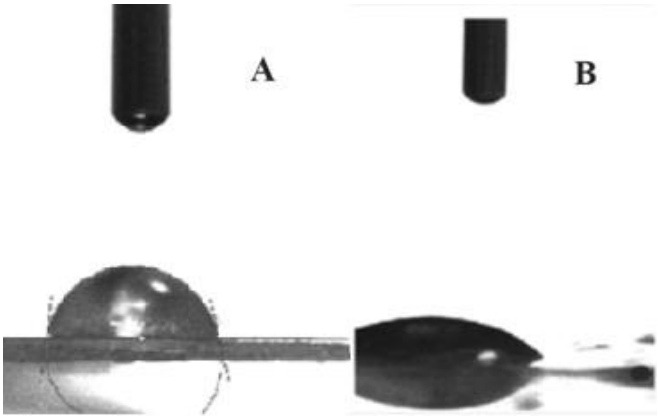

FIG.7 Photographs of the contact angles between deionized water droplet and glass surface: A) PLDLA and B) PLDLA-HAADH.

Conclusion. Finally, it was possible to confirm grafting of HAADH in PLDLA polymers by the protocol employed, observed in the results of wettability and swelling and indirectly in DSC and FTIR analysis. In this way, this work allowed the development of bioresorbable physical models,

with dimensions similar to PLLA coronary stents, coated with PLDLA with hyaluronic acid grafting.

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Disclosure. The authors report no conflicts of interest in this work.

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