World Academy of Science, Engineering and Technology
International Journal of Chemical and Molecular Engineering
Vol:12, No:7, 2018

Hydrogel Based on Cellulose Acetate Used as Scaffold for Cell Growth

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***Abstract*—**A hydrogel from cellulose acetate cross linked with ethylenediaminetetraacetic dianhydride (HAC-EDTA) was synthesized by our research group, and submitted to characterization and biological tests. Cytocompatibility analysis was performed by confocal microscopy using human adipocyte derived stem cells (ASCs). The FTIR analysis showed characteristic bands of cellulose acetate and hydroxyl groups and the tensile tests evidence that HAC-EDTA present a Young's modulus of 643.7 MPa. The confocal analysis revealed that there was cell growth at the surface of HAC-EDTA. After one day of culture the cells presented spherical morphology, which may be caused by stress of the sequestration of Ca^{2+} and Mg^{2+} ions at the cell medium by HAC-EDTA, as demonstrated by ICP-MS. However, after seven days and 14 days of culture, the cells present fibroblastoid morphology, phenotype expected by this cellular type. The results give efforts to indicate this new material as a potential biomaterial for tissue engineering, in the future in vivo approach.

***Keywords*—**Cellulose acetate, hydrogel, biomaterial, cellular growth.

I. Introduction

CELLULOSE acetate is one of the leading derivatives of cellulose and with great commercial importance. The degree of substitution (DS) can range from 0 to 3, being the commercial product with DS 2.5 one of the most important. Our research group has shown in recent years that the cellulose acetate with DS 2.5 is a great material to prepare hydrogels. By having many hydroxyl groups, it is capable of originate hydrogen bonding linked network, and has been recently shown possible of forming hydrogel through chemical crosslinking reactions [1]. Hydrogels can be defined as a stable three-dimensional network, in which due to their crosslinking properties is capable to absorb and/ or retain large amounts of water [2]. The physical properties such as flexibility, permeability and retention capacity of large volumes of water, make this material attractive for a large variety of pharmaceutical and biomedical applications [3]. However, for biomedical applications of hydrogels, as a biomaterial, more than their chemical and mechanical properties, it is also necessary that they be compatible with the cellular growth [4]. Because they are three-dimensional and absorb water, cell-compatible hydrogels, which is characterized by enabling specific molecular interactions with the cells, mimic the structure of the extracellular matrix, which has allowed important new discoveries with stem cell research, cancer biology and cellular morphogenesis [3]. The main characteristics that must be analyzed so that the hydrogels can be used as biomaterials are their capacity of bioadhesion, bioactivity, transport of biomolecules and mechanical properties [5]. Some biocompatible hydrogels have been used as scaffolds for cell growth in tissue engineering, as hydrophilic membranes for drug delivery and artificial tendon, skin and articular cartilage [3], [6]-[8].

ASCs have been widely used in association with biomaterials, since these cells have the ability to differentiate into mesodermal tissue cells such as chondrocytes, adipocytes and osteoblasts [9]-[11]. In addition, they have immunomodulatory capacity, being able to reduce the foreign body response generated by biomaterials [12]. Recently, these cells have been shown to have anti-fibrotic capacity, reducing the conversion of fibroblasts to myofibroblasts, which are responsible for the retraction of skin lesions [13].

Cellulose acetate is described as an excellent inert scaffold for cell culturing [14], [15]. The hydrogel based from cellulose acetate used in this work was crosslinked by ethylenediaminetetraacetic acid dianhydride (EDTAD) and our research group recently showed that this new hydrogel can increase 800% its own weight when immersed in water [1]. Such characteristics support the application of the hydrogel from cellulose acetate crosslinked with EDTAD (here named HAC-EDTA) as a material for biological purposes. Although some important properties of the HAC-EDTA gel have been widely discussed in the literature by previous work of our research group [16], this material has not been employed as a biomaterial. Complementary, specific analyses that have not yet held to the HAC-EDTA on earlier work involving the biocompatibility of hydrogels, have been carried out in a way unprecedented in this work. For example, cytocompatibility, confocal analysis and cell adhesion are original on the characterization of hydrogels. Additionally, other studies showed cellulose and its derivatives as highly biocompatible in in vitro models [17].

In our last work [1], one advantage that favors the use of

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HCA-EDTA is the low cost synthesis of this highly absorptive material, and herein, is further analyzed for others physicochemical and biological parameters. The originality of the characterization of this hydrogel presented here, showed that this material have strong hydrophilic features and ions release, together with cytocompatibility properties which is a promising candidate for the development and application as a biomaterial.

II. Materials and Methods

A. Materials

Rhodia® (France) produced the cellulose acetate with substitution degree of 2.5. The hydrogel is obtained through a homogeneous reaction involving hydroxyl groups present in cellulose acetate and anhydrides groups present in EDTA. The detailed method of synthesis of HAC-EDTA was previously published by our research group [1] and has a patent pending (BR1020120170434). In summary, this synthesis was carried out in a homogeneous solution phase that involves the dissolution of CA in DMF that outcome in the crosslinking of the hydroxyl groups with EDTA and triethylamine as catalyst. The HAC-EDTA was developed in a film form using casting techniques with two glass plates of dimensions 20 cm x 20 cm. After the synthesis process, the obtained film was cut into rectangular samples for DMA analysis or 0.6 cm circles for biocompatibility assays (Fig. 1). The hydrogel was also obtained in the form of finely divided powder. For that, soon after the synthesis, the hydrogel was fragmented with a mixer at high speed. Samples in powder form are required for the tests of DSC. The cell culture medium used was the Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% of fetal bovine serum (FBS) and 1% of antibiotic solution penicillin/ streptomycin, all purchased from Sigma-Aldrich (St. Louis, USA). The PBS containing TWEEN20® (PBS-T) were purchased from Life Technologies (USA). The ASCs was obtained from discarded liposuction in human plastic surgery and were kindly provided by Dr. Sara T. Olalla Saad (Department of Clinical Medicine, Faculty of Medical Sciences, State University of Campinas, UNICAMP, Brazil).

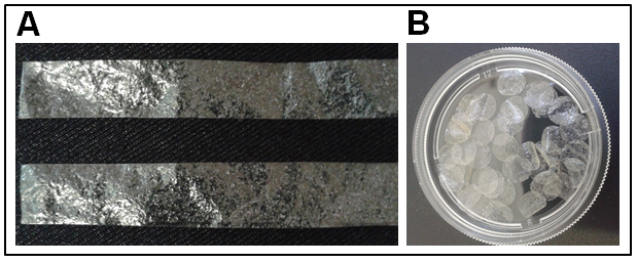

Fig. 1 (A) samples of HAC-EDTA obtained films for DMA analysis and (B) in circles format for cell culture assays

B. Differential Scanning Calorimetry (DSC)

Approximately 10 mg of powdered HAC-EDTA samples were heat treated from 35ºC to 45ºC at a heating rate of 5 ºC/min and then cooled to -100 ºC by purging N_2 at a temperature rate of 30 ºC/min. When the lowest cooling temperature was reached, the second heating ramp up to 45ºC was performed at the same rate of the first heating. All assays were made by DSC equipment model DSC-2910 (TA Instruments, USA).

C. Dynamic Mechanical Analysis (DMA)

The HAC-EDTA samples were processed as a film-shaped (15.45 mm x 7.67 mm x 0.16 mm), and the tension (stress-strain) was performed by dynamic mechanical analysis. Film samples were submitted to sinusoidal deformation of 240 µm by a constant frequency ramp of 1 Hz from 35ºC to 45ºC, at a temperature range increase of 1 ºC min^{-1}, using a strength standard according to the ASTM D 4065-2001. All samples were analyzed in tensile clamp holder in the DMA equipment model Q800 (TA Instruments, USA).

D. Compression Test

The hydrogel samples were submerged in water to swelling and then were cut in cube format with 50 mm in height and 50 mm in base. Compression tests were performed on a universal mechanical testing machine (CMT6104, MTS systems, China) with a load cell of 200 N at room temperature. A constant speed of 3 mm/min was loaded on the samples in the compressive mode and then the stress-strain curves of each sample were recorded. For each sample, at least three duplicates were tested to ensure good reproducibility (±5%).

E. Fourier Transform Infrared Spectroscopy (FTIR)

The HAC-EDTA films were analyzed in two situations: 1) dry films were immersed in water for 1 h to induce swelling and retrieved from water immediately before each analysis; 2) the as-received dry films previously stored under low moisture were analyzed to obtain the spectral bands characteristics. The assays were analyzed using PerkinElmer Spectrum 400 FT-IR Model Spectrum 400FT Mid-IR, at frequencies ranging from 400 and 4000 cm^{-1} and 16 scans.

F. Analysis of DMEM Adsorption of Ca^{+2} and Mg^{+2} Ions

The assays of Ca^{+2} e Mg^{+2} adsorption in DMEM were performed by the same hydrogel/ DMEM mass-volume proportion also used in the viability experiments. To proceed the test cell viability was used round samples of hydrogel in films of 0.035 g and DMEM volume of 200 µm, to study the adsorption of Ca^{2+} e Mg^{2+} ion and was necessary increase the mass of the hydrogel and the DMEM volume; that way, proportionately was used 5.25 g from hydrogel in film and 30 ml from DMEM. The studies were conducted following the procedure shown below:

1. The complexometric titration from DMEM before the contact with the hydrogel using EDTA standard solution 0.01 M. Was pipette 10 ml from DMEM and transferred for 250 ml Erlenmeyer, added 10 ml of ammoniacal buffer solution (NH_4Cl/NH_4OH) pH 10, around 100 ml distilled water, and drops of the Eriochrome black-T indicator, next was titrated with EDTA 0.01 M. The concentrations of Ca^{2+} and Mg^{2+} was calculated using:

$$C= [(Vg \times Ms \times M1)/Va]$$

where: C is the Concentration of Ca^{+2} or Mg^{+2} (g/L), Vg is the volume of EDTA 0,01M used on titration, Ms is the molarity of the solution of EDTA (0.01 M) is the molar mass of Ca or Mg and Va is the volume of the sample of DMEM used at titration.

2. The sample of hydrogel was immersed in DMEM with ambient temperature for seven days. Then the DMEM and the hydrogel were filtered and the remaining solution was analysed afterwards as described in topic 1.

G. Concentration of Ca^{2+} and Mg^{2+} Ions in the Culture Medium by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

ICP-MS was used to determine levels of Ca^{2+} and Mg^{2+} ions in the culture medium after cultivation of ASCs on HAC-EDTA. This test was performed to analyze the EDTA ions sequestration properties in the medium while under cell culture. The assay was performed after one, seven and 14 days in culture in HAC-EDTA disks. In longer culture times (seven and 14 days), new fresh medium was replaced every 48h. ICP-MS was also performed as control in the medium used in cultures of ASCs seeded on rounded glass coverslips. In addition, new fresh medium supplemented with 10% of FBS was also evaluated by ICP-MS.

H. Saturation of HAC-EDTA with Ca^{2+} e Mg^{2+}

The hydrogels were cut in disk shapes with 0.5 cm of diameter and then placed in saturated solution of calcium and magnesium under magnetic stirring for 5 hours. The discs were dried at room temperature and UV-instilled.

I. Scanning Electron Microscopy (SEM)

The hydrogel membrane samples were first swollen in distilled water and then dried at room temperature. The samples was not submit at critical point drying technique, but were cut in the middle and then sputter-coated with a thin layer of gold, the cured surfaces were then immediately observed using a MT-1000 scanning electron microscope (Hitachi, Japan).

J. In vitro Degradation Assay

The degradation assay consisted of submitting the material that simulates the characteristics of pH and body temperature with values next to the human body. The material was submitted at temperature of 37ºC immersed in PBS of pH 7.2. The film of HAC-EDTA was cut using a paper punch to obtain small disks, then it was freeze-dried for 72 h using a Freeze-Dryer Liotop, model K105, weighed individually and separately placed in Eppendorf tubes previous identified with the number of each sample and its initial mass, containing 1.5 ml of PBS. All tubes were placed in racks and put into the microprocessor controlled water bath (model Q215M, Quimis). This assay took 10 months, untouched, in the same conditions and after this time, the samples were freeze-dried and weighed again.

K. Laser Scanning Confocal Microscopy (LSCM)

Cells were cultivated over HAC-EDTA or glass coverslips for one, seven and 14 days prior to LSCM experiments. After fixation with 4% PFA for 30 min, cells were washed in PBS-T and permeabilized with 0.1% Triton X-100 for 15 min. After PBS-T baths, samples were immersed in 2% BSA for 1 h followed by PBS-T washes, than were incubated in a solution of 1:200 phalloidin conjugated with Alexa 647 for 40 min. The unbounded phalloidin was released after several baths of PBS-T and samples were stained with Fluoroshield containing DAPI solution (Sigma-Aldrich). The 405 and 638 laser lines were used in PMT mode to detect the wavelengths in the range of DAPI and Alexa 647 emission spectra, respectively. All samples were analyzed by the confocal microscope, model TCS SP8 (Leica Microsystems, Germany), and signals were detected and digitized by LAS AF software.

III. Results and Discussion

A. Thermal Performance of the Material by DSC

The DSC analysis did not present any change during the first heating, notwithstanding a slight difference was detected in the second heating at the temperature curve, due to the endothermic peak identified by the Tg value in the region of 13.8ºC (Fig. 2 (A)). This value represents the mobility of polymeric chains during the amorphous phase of the HAC-EDTA, in the moment when the switchover of an ordered phase to a more flexible and disordered one occurs.

B. Dynamic Mechanical Analyzer (DMA)

DMA technique allows meeting not only the mechanical behavior of materials, but also the structure, morphology and its viscoelastic behavior. The analysis of HAC-EDTA film was performed at a temperature ramp of 35-45ºC and the material presented a maximum rupture force of 14.5 MPa and deformation of 2.3% (Fig. 2 (B)). The hydrogel used presented a Young's modulus of 643.7 MPa. Considering the analysis results, this material would be interesting as a cutaneous dressing, since the Young's modulus of human skin ranges from 4.6 MPa to 20 MPa [18] or as substitute of cartilage which has module between 0.45 MPa to 0.80 MPa [19] and the Young's modulus of the tendon is <1000 MPa. According to [20], the human skin presents tensile resistance values between 2.5 MPa and 16 MPa, while Hansen and Jemec [12] confirmed that human skin presents elongation and deformation near to 70%.

The tensile resistance observed by HAC-EDTA of 14.5 MPa, and besides, the fact that the HAC-EDTA hydrogel is transparent allows for injury monitoring and it absorbs water, maintaining the contacted area hydrated [1], which could make this material a future candidate for applications such as a skin dressing.

C. Compression Test

Fig. 3 shows representative compression stress x strain curves in compression mode for swollen hydrogel and Table I shows the main results obtained for the three samples analysed.

The Young's modulus or modulus of elasticity is a mechanical parameter that provides a measure of the stiffness

of a solid material. It is a fundamental parameter for the engineering and application of materials because it is associated with the description of several other mechanical properties. Table I shows that the modules are dependent on the area of the material submitted to tests.

The behavior of HAC-EDTA is typically the same to several others described in the literature. Wei et al. [21] present values of compression stress (kPa) in the order of 20 to approximately 68 to hydrogels based on PNIPAm/ TO-CNF. The stress x strain curve in compression mode features a small increase of the load supported by the hydrogel in the first moments of the test, followed by a sharper increase at strain higher than about 60% (Fig. 3). As the hydrogel content increased, the maximum stress and toughness of the hydrogel consistently increased [22]. The hydrogel does not fail on compression by a shattering fracture, so in that case, the compressive strength showed to be an arbitrary one, indicating complete failure of the material. The mechanical compression test was taken to the hydrogel resistance limit. This limit was reached with about 80% deformation (Fig. 3). After this compression limit, the hydrogel suffered irreversible plastic deformation and HAC-EDTA has presented a compacted disk shape after the resistance limit. Additionally, no visible fracture was observed in the material after plastic deformation [21].

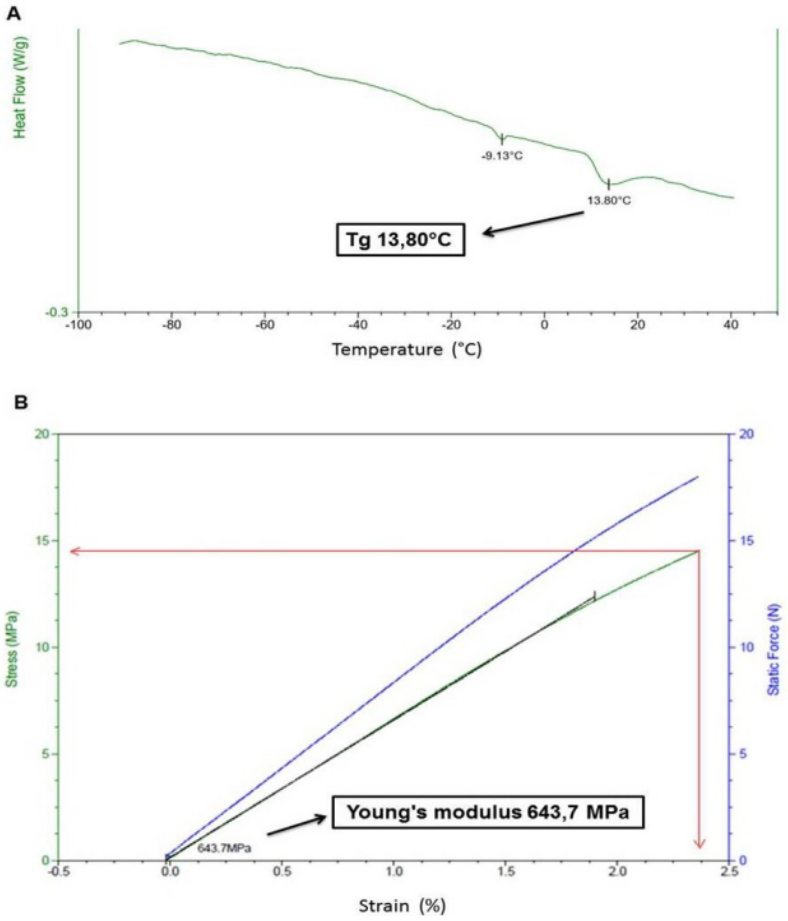

Fig. 2 (A) DSC curve of the second warming cycle from 25ºC to 45ºC with heating ramp of 5ºC/min of HGAC. (B) Stress-Strain of HAC-EDTA by DMA at the temperature ramp of 35ºC to 45ºC

TABLE I
MODULUS AND PEAK STRESS (MPA) FOR STRAIN X STRESS ANALYSIS OF HYDROGEL IN COMPRESSION MODE ASSAYS

Compression Specimen	Area (mm^2)	Modulus (MPa)	Peak Stress (MPa)
1	109.8	66.46	7.55
2	130.5	46.20	5.61
3	97.8	89.47	14.96
Mean	112.7	67.38	9.37
Std. Dev.	16.5	21.64	4.93

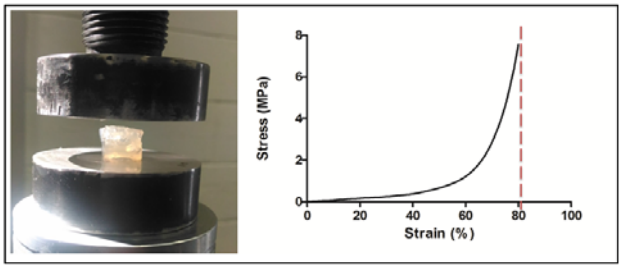

Fig. 3 Typical compressive stress began to slowly increase with strain, followed by a sharper increase at strain higher than about 60%

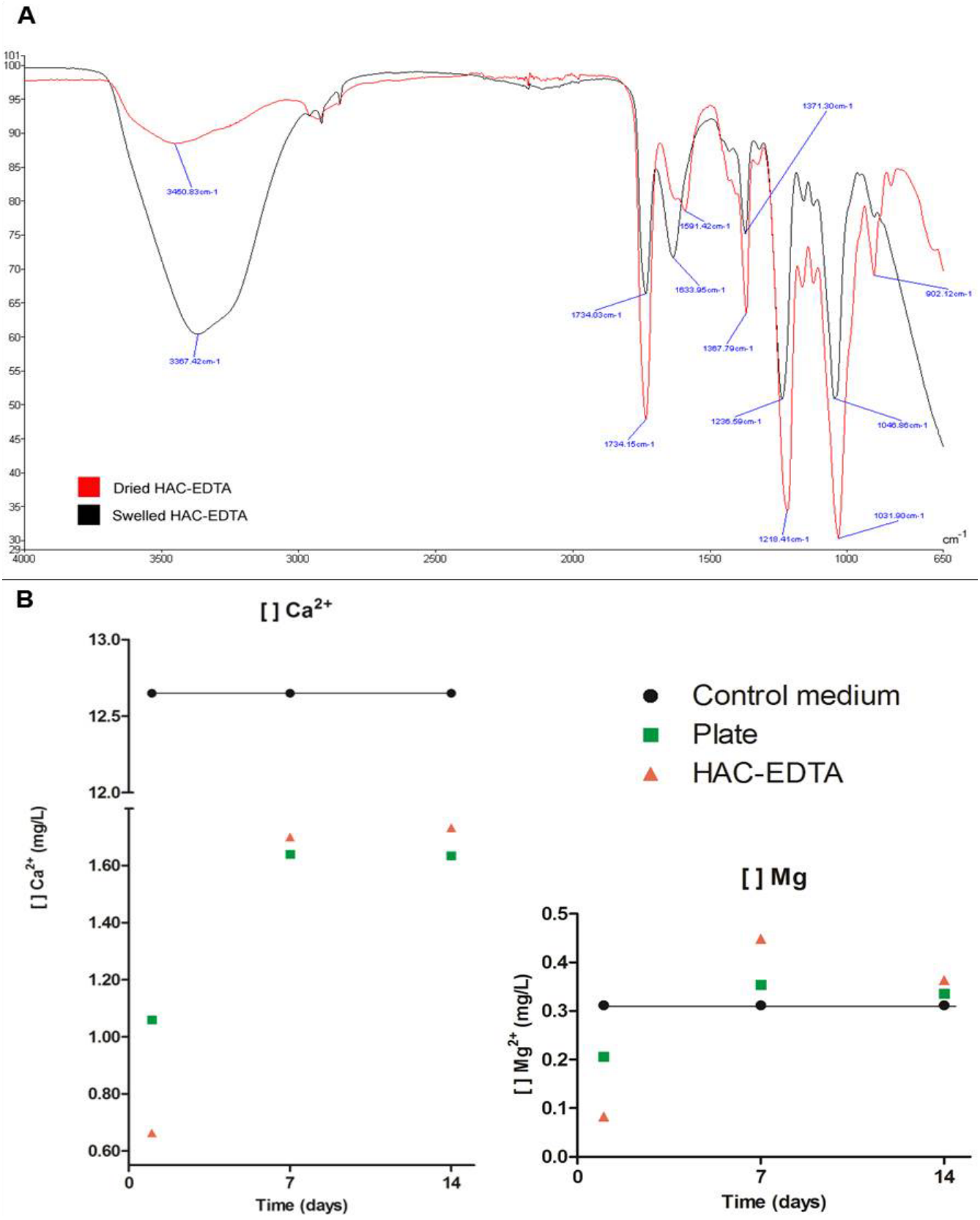

Fig. 4 (A) FTIR spectra for Dried and Swelled HAC-EDTA. (B) ICP-MS of Ca^{2+} and Mg^{2+} ions in the culture medium after cultivation of ASCs on HAC-EDTA. The plate in the figure captions represents the positive control using polystyrene (PS) plate and ASC cell, the ions concentration was measured after one, seven and 14 days. The medium only refers to the unused medium for culture assays and was supplemented with 10% of FBS

D. Fourier Transform Infrared Spectroscopy (FTIR)

The swelled HAC-EDTA presented characteristic bands of cellulose acetate, also presenting an intense band at 3470 cm^{-1}, attributed to stretching of hydroxyl groups (Fig. 4 (A)). Typical bands at 2940 cm^{-1} characteristics of axial deformation vibration of bonds C-H aliphatic can be observed. The intense band at 1747 cm^{-1} refers to vibrations of the stretching bands C=O.

The band situated at 1370 cm^{-1} is relative to deformations –CH3, and the band at 1236 cm^{-1} is the asymmetric distension of bonds C-O-C, which is esters characteristics. The band around 1031 cm^{-1} is related to vibrations of bonds C-OH, which has alcohol characteristic bands in the structure [18], and is listed in Table II.

It is evident that in both situations (the hydrogel when hydrated or dried), the spectrum only differs in the intensity of bands. Thus, the following situation was verified: when HAC-EDTA was swollen, the bands that refers to the stretching vibrational of OH suffer an increase of around 3400 cm^{-1} and 1635 cm^{-1} in intensity, resulting in water absorption. The FTIR analysis demonstrated that the characteristic bands of the HAC-EDTA enabled it to grab large amount of water, due to OH stretch of hydrophilic groups. The usage of EDTA in the composition of biomaterials was recently discussed by [1], which considers the crosslinking with EDTA as an additive amphoteric characteristic, and that can correspond to an increased swelling property of HAC-EDTA.

TABLE II
CHARACTERISTICS FTIR BANDS FOR CELLULOSE ACETATE (CA) AND HAC-EDTA

Characteristics bands	Absorption Bands		
	*CA [15]**	*HAC-EDTA*	
		*Dried**	*Swelled**
OH stretch of hydrophilic groups	3470	3450	3367
Axial deformation vibration of aliphatic C-H bonds	2940	**	**
Angular deformation of the C = O bond	1747	1734	1734
Deformations $–CH_3$	1370	1367	1371
Asymmetrical stretching of the C-O-C bond	1236	1218	1236
Binding C-OH vibrations	1031	1031	1046

*Values are expressed in cm^{-1} ** No significant bands detected

E. Concentration of Ca^{2+} and Mg^{2+} Ions in the Culture Medium

The levels of Ca^{2+} and Mg^{2+} ions in the culture medium after cultivation of ASCs on HAC-EDTA were determined using complexometric titration from DMEM before and after the contact with the hydrogel, and we also used the Inductively Coupled Plasma-Mass Spectrometry (ICP-MS).

Complexometric Titration with EDTA was used in determination of concentration of Ca^{2+} and Mg^{2+}. It is interesting to mention that Complexometric Titration is simple and also allows the quantification of both ions at the same time. This quantification of both ions is possible since the constant complexes formation of the EDTA with Ca^{2+} and Mg^{2+} are very close [1], [23].

To find a best way to characterize the concentrations of Ca^{2+} and Mg^{2+} on DMEM, first was performed consultation of the manufacturer data of DMEM, and from that, it was evident that DMEM have 19.7 mg of Mg^{2+} ion and 82.3 Ca^{2+} ion per liter of DMEM.

The results of determinations of Ca^{2+} and Mg^{2+} in our work were compared to the data of the manufacturer to prove the accuracy of our experiments. The amount of concentrations of Ca^{2+} and Mg^{2+} (102 mg/L), 80.7% was of Ca^{2+} and 19.3% of Mg^{2+}. That is, every liter of DMEM presents 2.054 $x10^{-3}$ mols of Ca^{2+} ion and $8.11X10^{-4}$ mol of Mg^{2+}. This way, 71.7% to the volume of EDTA consumed in titration of DMEM to complex the Ca^{2+} ion and 28.3% to complex the Mg^{2+} ion. The analogy suggests the following results:

The DMEM analyzed present concentrations of 77.6 mg of Ca^{2+}/L and 18.6 mg of Mg^{2+}/L, very similar values of the theoretical values. In this way, the method used was workable for determining the concentrations of Ca^{2+} and Mg^{2+} ion on DMEM.

The results of the analyses of Ca^{2+} and Mg^{2+} after the immersion of the hydrogel was surprising, because it indicates that all Ca^{2+} and Mg^{2+} ion was absorbed by the hydrogel.

The titration, after the addition of the Eriochrome black-T indicator, the coloring changed immediately to dark blue, even before adding any drops of EDTA 0.01 M, which shows that all Ca^{2+} and Mg^{2+} was absorbed by HAC-EDTA.

A very interesting result can be reported in relation to cell culture in the presence of the hydrogel. It is well known that cells need minimal concentrations of nutrients to reproduce. In contact with the culture medium, the hydrogel absorbed a large amount of the nutrients present in the culture medium. Previous work by the research group has shown the amphoteric character of the hydrogel and its enormous capacity to absorb ions [1]. Thus, the availability of nutrients to the cell growth was insufficient for the development of the cells, which explains the lack of cellular activity and the death of some cells. The adsorption of ion by HAC-EDTA can be taken into account because, as can be seen from the results of biocompatibility, after the first 24 h of cell exposure, at the surface of the hydrogel it is possible to observe the gradual decay of cell activity by LSCM; thus, the adsorption of Ca^{2+} and Mg^{2+} ion could justify this result.

After one, seven and 14 days in cell culture in HAC-EDTA, the medium was collected and analyzed (Fig. 4 (B)). As controls, it was used the ASCs grown on the polystyrene containing cells and DMEN culture medium. In another, PS plate was added to only culture medium without cells. In this case, the PS plate containing cells is the positive control and negative control is PS without cells. We observed that there were calcium and magnesium ions depletions in HAC-EDTA and in the control after one day in culture, and that such depletion was 1.7-fold higher in the control as compared to HAC-EDTA. After seven and 14 days in culture, there was a recovery in the concentration of these ions, while HAC-EDTA presented a slight increase in such retrieval which is indicative of the saturation of Ca^{2+} and Mg^{2+} by the HAC-EDTA. The initial sequestration of Ca^{2+} and Mg^{2+} in the culture medium

after one day while in contact with HAC-EDTA was later recovered, and followed by a slight increase of these ions after seven and 14 days. After these times, the ICP analysis detected an increase in Ca^{2+} and Mg^{2+} in the medium that can be directly related to a late ions release. Such characteristics give efforts to additional investigations as a device applied to the absorption of hydrophilic drugs followed by its controlled release in a delivery device model. Interestingly, it is known that cell spreading occurs only in the presence of Ca^{2+} and Mg^{2+} [23], and the initial ions sequestration observed by the HAC-EDTA may have deprived the cells of these essential salts and affected the cells morphology.

F. Saturation of HAC-EDTA with Ca^{2+} e Mg^{2+}

The cell viability assay using the Ca^{2+} e Mg^{2+} saturated films showed that during the first 24 hours, the cell adhesion is better than control (hydrogel without saturation) at the same time, which corroborates with the titration result, where the hydrogel removes the Ca^{2+} and Mg^{2+}, making both cell adhesion difficult. However, in the cytotoxicity results before saturating the hydrogel, it shows that only after the seven day time, the cells have better adhesion and cellular activity. This can be justified by the exchange of culture medium every two days, where a new complete culture medium is replaced by the culture medium that has already interacted with the hydrogel, causing Ca^{2+} and Mg^{2+} saturation over time to occur culture. This entire process can be easily solved by saturating the hydrogel films prior to cell viability assays (Fig. 5).

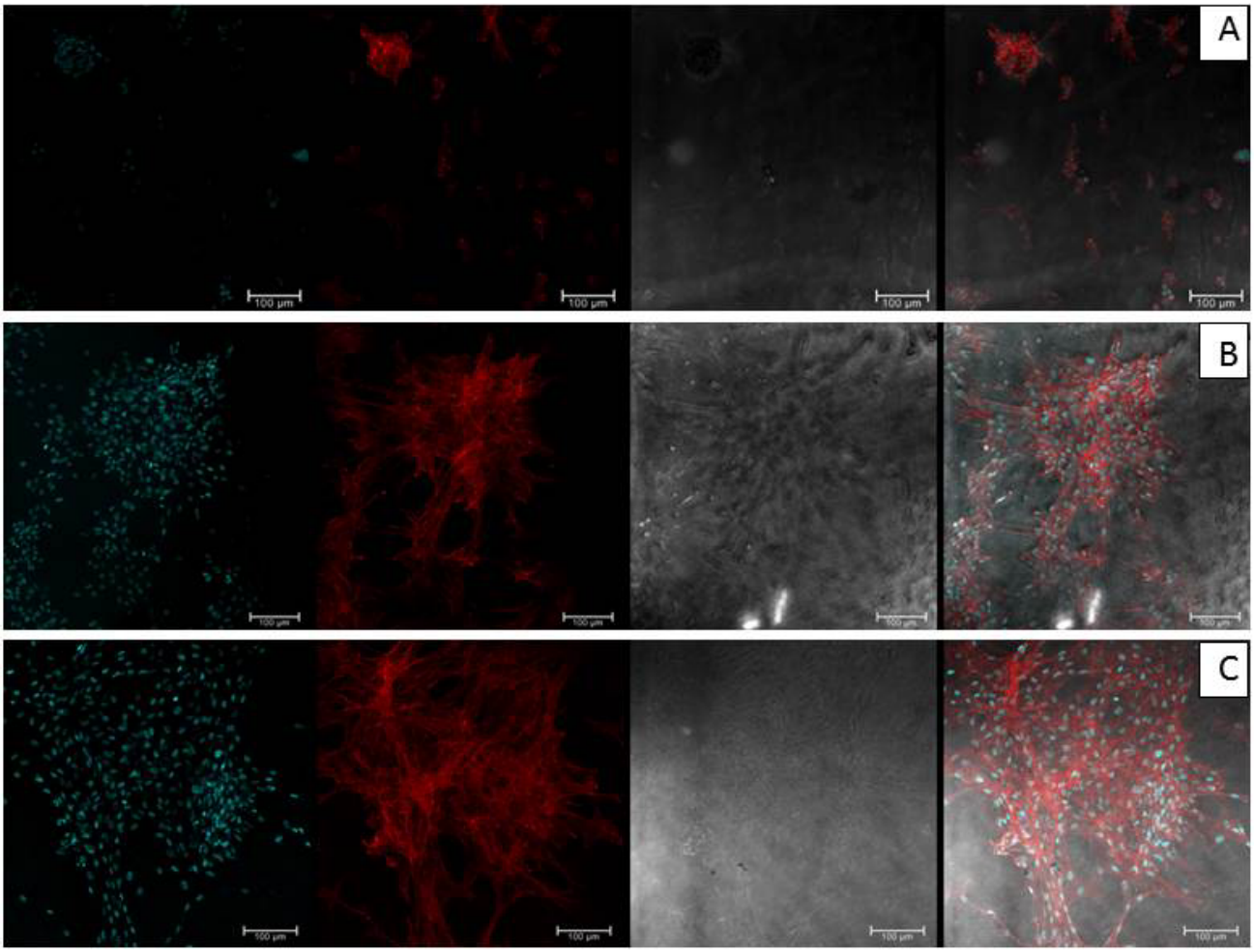

Fig. 5 LSCM of ASC cells adhered in the HAC-EDTA in culture times of 1 and 7 days. (A) Hydrogel without saturation of Ca^{2+} e Mg^{2+} on first day of culture, that represents difficult of cells to adhesion at the material surface. (B) After saturation of Ca^{2+} e Mg^{2+} the cells have better adhesion in the material, presenting greater amount of cells in the same time of one day, and (C) Represents the time of seven days, where the cells increased in quantity and continue to have adequate cell type morphology

G. In vitro Degradation Assay

Samples submitted to in vitro degradation test were lyophilized and weighed. The results did not present statistically significant change concerning the sample mass before and after the test. This indicated that during the period of 10 months, the material did not suffer any degradation process even under continuous immersion in PBS at 37ºC. The not significant weight loss is evidence of the material stability (data not shown).

H. Scanning Electron Microscopy (SEM)

The cross-section surfaces of the hydrogel membrane were examined using scanning electron microscope and their interior micrographs are presented in Figs. 6 (B)-(D). All samples exhibited irregular three-dimensional surface without porosity structure with fairly dense and relatively smooth surfaces. The section of hydrogel that was cut had flaws caused by the formation of bubbles during the synthesis of the material, which presented irregular structures, different from the surface and the sectioned part. Although the material did not present porosity and was not prepared at the critical point, it was not able to interfere in cell adhesion (Figs. 6 (E) and (F), Fig. 7), which shows that the material presents interesting characteristics as biomaterial.

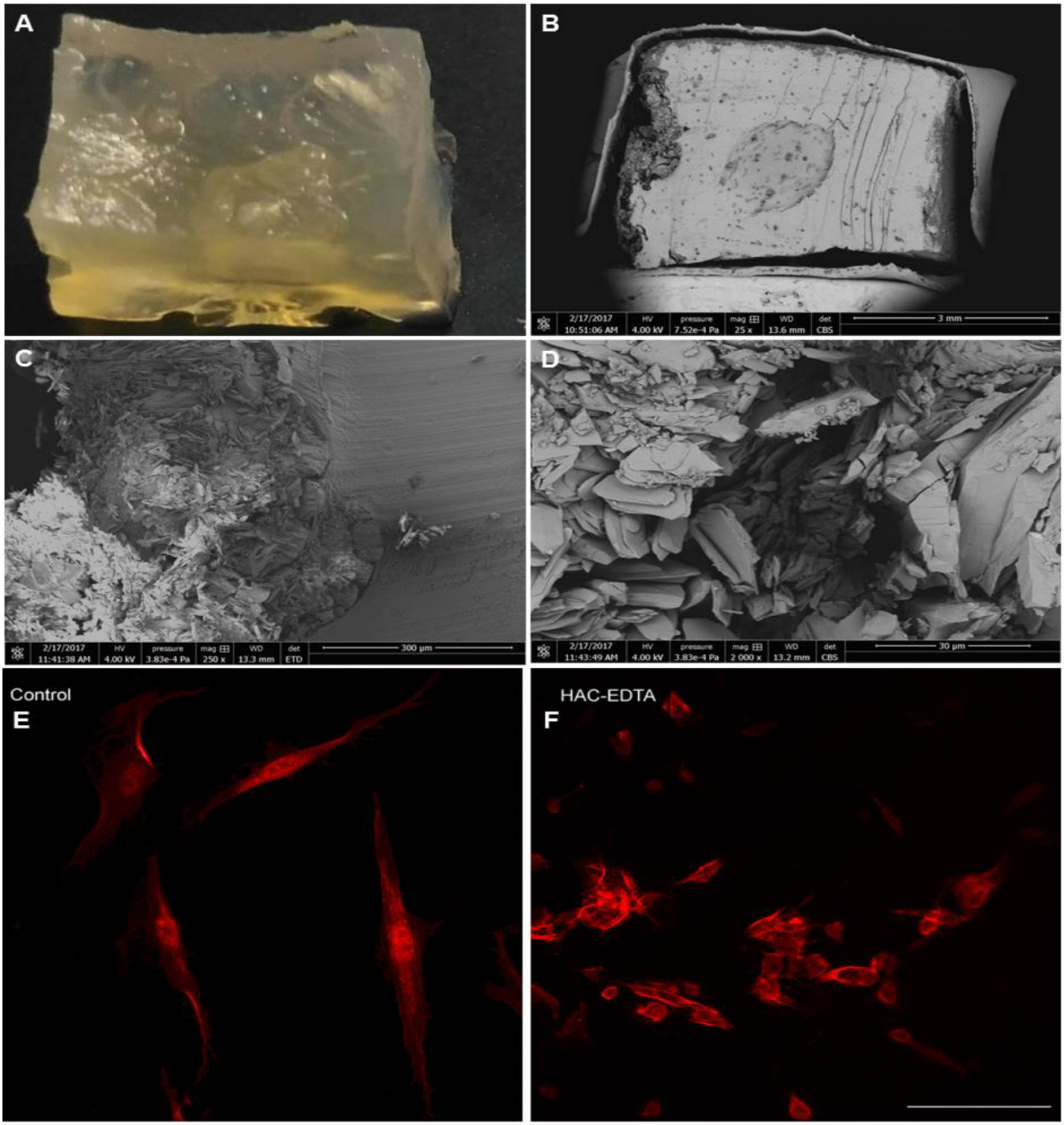

Fig. 6 (A) HAC-EDTA dried at room temperature. (B) Membrane in cubic form, cut in half, having on its right side deformation caused by the formation of beads in the material during the synthesis. (C) Surface of the hydrogel with partially smooth characteristics. (D) In the cut length presents a smooth appearance, while in the deformation it is possible to observe an disordered structure. (E) Confocal images of the osteoblast-like cells (labeled in red by phalloidin-TRITC) adhered to glass coverslips (control) or to (F) HAC-EDTA. Bars (control) 15µm and (HAC-EDTA) 250µm

I. Morphology of ASCs by Laser Scanning Confocal Microscopy (LSCM)

The morphology of ASCs in the hydrogel was evaluated by LSCM after one, seven and 14 days in culture. After one day, the cells presented low adhesion observed due to roundish cytoskeleton morphology and the loss of actin stress fibers while some cells presented pyknotic nuclei, which is not consistent with viable cells morphology (Fig. 7). Interestingly, after seven and 14 days in culture in the hydrogel, the cells were highly spread and typical actin stress fibers were observed.

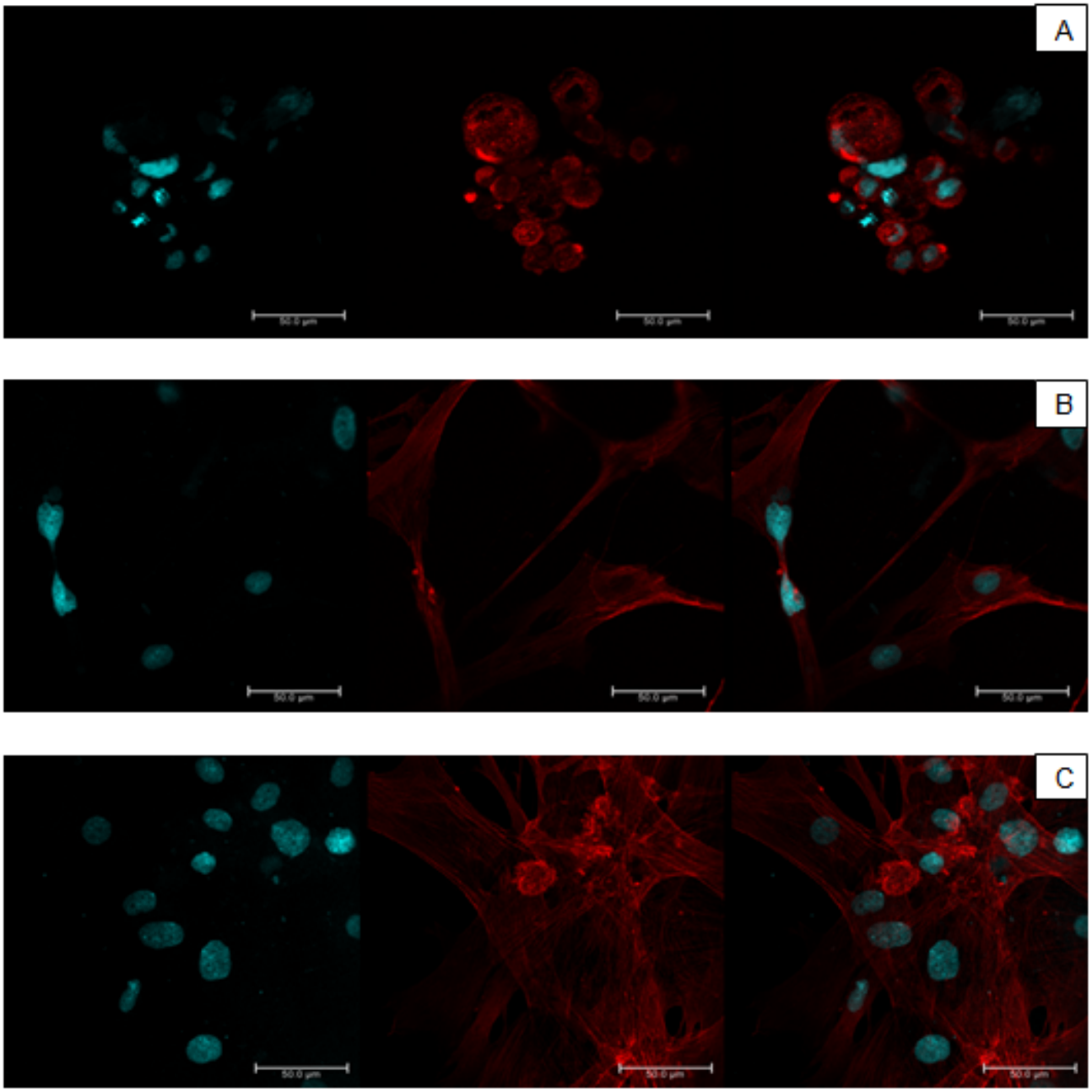

Fig. 7 LSCM of ASC cells adhered in the HAC-EDTA in different culture times. The nuclei stained with DAPI (blue) and the actin labeling by phalloidin conjugated with Alexa 647 (red) revealed the ASC morphological pattern when adhered in the HAC-EDTA substrate. Where: (A) one day, (B) seven days and (C) 14 days

In Fig. 7 (A), the remarkable distinct cell morphology of one day in culture compared to seven and 14 days might be related to initial cell death process. After one day in culture, the cells did not exhibit the expected morphology, and presented small rounded shape. The ASCs also showed some pyknotic nucleus and unorganized cytoskeleton, so the cells probably have undergone a period of stress in these first hours. However, after seven and 14 days, the release in Ca^{2+} and

Mg^{2+} was also followed by the recovery of cells spreading and proliferation as shown by LSCM. The initial low cell adhesion followed by its late recovery, reinforces the importance of these ions to cell attachment and also directly correlate the cell viability to the release activity of HAC-EDTA. This cytocompatibility assay is an initial step in the analysis of the toxicity of biomaterials. Because, to date, there are no standard parameters for predicting the behavior of cells in biomaterial [24]. The presence of stress fibers in spread cells indicate strong adhesion and is the main signal that cells viability is established [25]. Therefore, we can say that the hydrogel was cytocompatible after it starts releasing ions that consequently favored the adhesion, proliferation and spreading of ASCs.

It has been demonstrated that extracellular Mg^{2+} and Ca^{2+} deficiency reduces the proliferation of human ASCs cells and is directly related to changes in cell membranes receptor channels involved in assorted functions, such as the cell adhesion [26]. Thus, the relative rounded shapes observed by LSCM could also be considered an evidence of an unbalanced receptor channels due to the low extracellular Mg^{2+} and Ca^{2+} levels as reported by previous literature [26]. Thus, the indication of HAC-EDTA as a biomaterial application should be directed after this material reaches an ionic sequestration/ release equilibrium.

Mechanical, chemical and biological analyzes of the material demonstrated good possibilities of this material as a candidate for a medical device applied to skin healing.

IV. Conclusions

The results showed that HAC-EDTA has excellent mechanical resistance, while it also has a low cost production. It also does not degrade in short periods of time and the previous known swelling properties showed that HAC-EDTA sequestrate Ca^{2+} and Mg^{2+} ions, which is followed by a late release of these ions. Initial cell growth had low viability, while in long-term cultures, the cells recovered growth and spreading. Regarding the hydrophilic properties, the release behavior of this material and the cell biocompatibility indicate this material as a strong candidate for cutaneous curative.

Acknowledgments

The authors declare that there is no conflict of interest regarding the publication of this paper. We thank to the Profs. Drs. Elaine S. Zacarin, Fabio de Lima Leite and Monica Jones for granting the use of the LSCM at UFSCar-Sorocaba-SP, and the technical staff of the Biomaterials Laboratory/PUC-SP. Additional thanks to Prof. Dr. Celso Aparecido Bertran (Unicamp) for the support on ICP and SEM analysis.

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