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Special Issue: Bioactive Surface Functionalization

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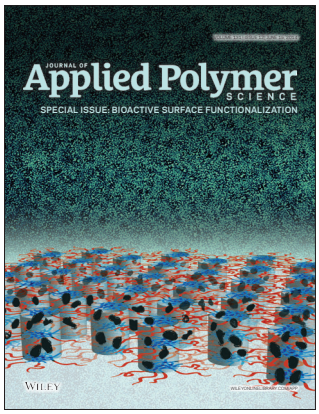

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Fibroblast Viability and Inhibitory Activity Against *Pseudomonas aeruginosa* in Lactic Acid-Grafted Chitosan Hydrogels

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ABSTRACT: Chitosan (Ch) from biologically obtained chitin by lactic acid fermentation process was successfully grafted with lactic acid following three different procedures, direct D,L-lactic acid attachment with and without the aid of *p*-toluene sulfonic acid catalyst and by ring-opening of L-lactide. The produced materials behave as hydrogels responsive to pH changes and were characterized by X-ray diffraction analyses, swelling behavior, erosion, contact angle, and thermally. In addition, mechanical studies in films of the synthetized materials demonstrate that the increase in grafting improved the mechanical properties in terms of tensile strengths. All the grafted Chs showed inhibition of the pathogenic bacteria *Pseudomonas aeruginosa*, which corroborate the preservation of antimicrobial activities in the modified Ch. The study on cell attachment and viability of fibroblasts onto the grafted Ch films evidenced that regardless of the low mechanical improvement in the lowest incorporation of lactic acid (31%), it rendered the materials with the highest adherence and viability of fibroblasts. Nonetheless, lactic acid incorporation enhanced cell viability in all synthetized materials as compared to native Ch. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40252.

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INTRODUCTION

Chitin and its main derivative Chitosan (Ch) have received much attention for biomedical applications, such as in tissue engineering (TE) for wound healing. Ch has advantageous characteristics such as renewability, biocompatibility, biodegradability, bioabsorbability, antimicrobial, and hemostatic activities.[1] Studies *in vivo* showed that angiogenesis and migration of neutrophils were the result of cytokine IL-8 release from fibroblast induced by chitin derivatives.[2] In a related work, Ch and their degradation products, oligomers and monomers, were reported by Minagawa et al.[3] as accelerators of wound healing. These authors observed many activated fibroblasts at the wound using Ch derivatives, and increase on the activity of collagenase, enzyme related to wound remodeling. Ch promotes granulation tissue, re-epithelization, and enhances vascularization. This polysaccharide is a continuous supply of chito oligomers to the wound that stimulate correct deposition, assembly, and orientation of collagen fibrils, which are incorporated into the extracellular matrix components. Moreover, owing to its antimicrobial activity, Ch has also been exploited for prevention of infection.[4]

However, despite of the reported benefits, poor mechanical properties are often associated to this polysaccharide, which might restrict their potential as biomaterial. A way to circumvent this drawback is the grafting of Ch to modify the mechanical properties.[5] In this regard, the biocompatible and naturally resourceable lactic acid (LA) has shown to improve mechanical properties when linked, often randomly, to many polymer backbones.[5,6] The stereoisomers of LA are precursors of polylactides (PLA), which have been used in TE as three-dimensional porous scaffolds for bone regeneration; in orthopedic surgery for fracture fixation, and in oral implants, mainly in the form of plates, nails, screws, and fibers.[7]

Additional Supporting Information may be found in the online version of this article.

Recently, we have shown the advantages of our biological process to attain chitin and the further use of a freeze-pump-thaw deacetylation method for chitosan preparation.[8] The attained biological chitin presented low depolymerization and high degree of acetylation (DA). Further deacetylation process of the biological chitin produced Ch with block copolymer structure in high crystallinity, which is sought when biomedical applications. The aim of this work was the preparation and characterization of potential biomaterials based on hydrogels of LA-grafted onto our Ch from biological chitin to study human dermal fibroblasts (hDFs) attachment and viability, and growth inhibition of nosocomial pathogenic bacteria.

MATERIALS AND METHODS

Materials

Chitin was obtained by lactic acid fermentation of shrimp wastes (*Litopenaeus vannamei*) as previously reported.[8] Ch with $M_v = 9.59 \times 10^5$ g mol^{-1} and $DA = 9.8\%$ was obtained by heterogeneous deacetylation under nitrogen atmosphere. Racemic D,L-lactic acid (D,L-LA) (85% aqueous solution) was obtained from JT Baker (Mexico). L-lactide was supplied by Sigma-Aldrich, recrystallized from methanol (technical grade) and stored at 5°C. Triethanolamine (TEA) and *p*-toluene sulfonic acid (*p*-TSA) were obtained from Sigma Aldrich.

Ch Grafting Procedures

Grafted copolymer of D,L-LA onto Ch to attain sample A_{30} was produced as follows: 40 mL of a Ch (1 g) solution in D,L-LA (0.5*N*) was mixed with 160 mL of D,LL-LA (0.3*N*) and the mixture heated at 80°C for 2 h.[6] A second material (C_{75}) was synthesized following the same procedure but adding 1% (w/w) of TSA. Reaction was purged with nitrogen and conducted at 80°C for 24 h.[9] Then, solutions were poured into petri dishes placed under vacuum at 80°C for 3 h and further dried at 60°C for 6 h. The resulting films were purified with acetone for 18 h. Ch-*g*-poly-L-lactide (B_{55}) was prepared by placing the chitosan (1 g) solution (D,Ll-LA 0.5*N*) in a flask under nitrogen atmosphere, to which L-Lactide (4 g) and TEA (0.5 mL) were added. Reaction was conducted at 80°C for 24 h.[9] Reaction product was precipitated in cold acetone (1/10 v/v), separated by centrifugation, dried, rehydrated, and finally placed in the petri dishes to form the film, which was purified with acetone.

Product Characterizations

Viscosimetric molecular weights (M_v) were calculated by an Oswald viscosimeter using the Mark-Houwink-Kuhn-Sakurada ($\alpha = 0.85$ and $K = 1.38 \times 10^{-5}$ L g^{-1}) equation. Ch and grafted samples were dissolved in acetic acid (0.3*M*) and sodium acetate (0.2*M*) at 25°C under stirring.[8] Soluble matter was determined gravimetrically by dissolving the samples (100 mg) in acetic acid solution (0.1*M*) for 24 h at 25°C. Then, solutions were filtered through filter paper (Whatman 40), dried overnight at 100°C and weighted at room temperature. Results are reported as weight percentage of soluble matter. DA was determined by integration of characteristic signals in the ^{1}H NMR spectra recorded in D_2O with 10% HCl in a Bruker AVANCE-III 500 (Germany) spectrometer.[10] Degree of grafting in percentage (% DG) of samples was calculated from integration of the characteristic methyl group of lactyl units and the massive of Ch signals in the ^{1}H NMR spectra according to eq. (1).

$$\%DG = \left(\frac{1/3A_{CH_3}}{1/6\sum_2^6 A_{Hi} + 1/3A_{CH_3}} \right) \times 100 \quad (1)$$

Infrared spectra were acquired in an ATR-FTIR (Perkin Elmer 100, UK). Thermogravimetric analysis (TGA) was conducted in a TA Instruments Hi-Res TGA 2950 at a heating rate of 10°C min^{-1} under nitrogen atmosphere. Crystallinity index (I_{CR}) and apparent size of crystallite (D_{ap}) were determined in the X-ray diffraction spectra recorded in a Siemens D-5000 diffractometer with an index of radiation of CuKα and a wavelength $\lambda = 1.5406$ Å, in the range of $2\theta = 3$ to 80° with steps of 0.02°. I_{CR} values were obtained by the intensity of the peak (110) about $2\theta = 20°$ (maximum intensity) and $2\theta = 16°$ (intensity of the amorphous peak) according to eq. (2).[11]

$$I_{CR} = \frac{I_{110} - I_{am}}{I_{110}} \times 100 \quad (2)$$

D_{ap} values were determined by eq. (3) from the diffraction pattern profiles.

$$D_{ap[110]} = \frac{K.\lambda}{\beta_0 \cos\theta} \quad (3)$$

where $K = 0.9$, β_0 (rad) is the width at half the height of the crystalline peak, and 2θ is the scan angle (110) diffraction line.

Mechanical properties in terms of tensile strength on extension were determined according to ASTMD882-97 (2003) and the tensile strength on puncture as described by Gontard et al.[12] in a SINTECH 1/S (MTS) apparatus with a load cell of 100 N. Samples were conditioned to 44% RH at 25°C before analyses. Percent of swelling (%H) was determined by introducing dry films of 1 cm^2 in 5 mL of Dulbecco's modified eagle medium (DMEM) at 37°C and recording the weight of the sample every 24 h [eq. (4)]. Erosion percentage (%E) was determined during 14 days in DMEM where test samples were withdrawn every 48 h, rinsed with deionized water and freeze-dried, and thereupon weight was recorded [eq. (4)].

$$Y(\%) = \frac{X_f - X_0}{X_0} \times 100 \quad (4)$$

where Y is swelling or erosion, X_f is the final weight of the dry sample, and X_o is the initial weight of the dry sample.

Water contact angle on films was determined in areas of aproximately 1.5 cm^2. One microliter of deionized water was dropped onto the surface of film and observed with a side-illumination horizontal light microscope Intel Qx3 (Intel Corporation). Contact lengths (b) and heights (h) of the water droplet were measured by ImageJ 1.41o software (National Institutes of Health). Contact angle was calculated according to the eq. (5).[13] Analyses

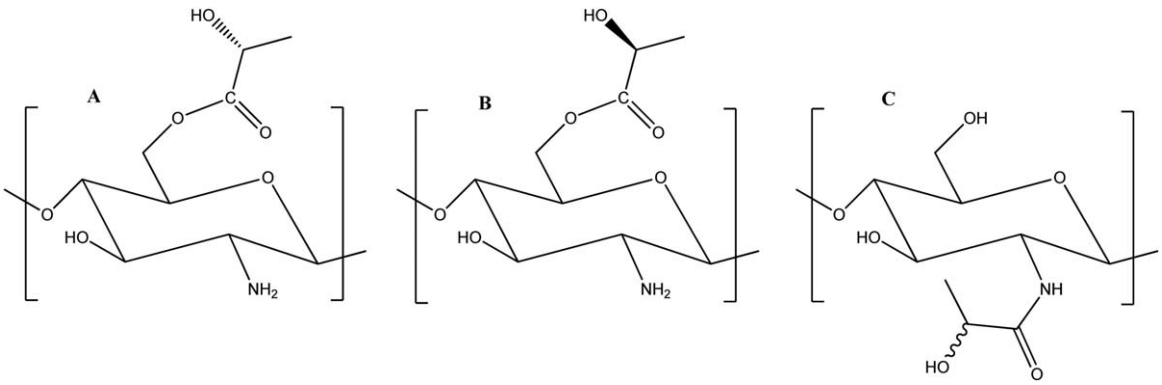

Figure 1. Proposed molecular structures of the repeat units by ester formation for (A) D-lactyl and (B) L-lactyl units grafted onto chitosan and by amide bond formation (C).

were conducted by quadruplicate, in three different random locations on each surface.

$$\theta = 2\tan^{-1}\left(\frac{2h}{b}\right) \quad (5)$$

Fibroblast Attachment and Viability

hDFs were isolated from adult skin, normally discarded tissue after routine plastic surgery of healthy patients and after patient consent. Adipose tissue was removed and the dermis was isolated from epidermis and digested with trypsin and collagenase. Cells were cultured in DMEM supplemented with 10% of fetal bovine serum and 1% of penicillin and streptomycin (Gibco) and were incubated at 37°C, 80% relative humidity and 5% of CO_2 for 1 week.[14] Films were sterilized with UV light for 10 min in a cross-linker (BLX-254 Vilber Lourmat, Germany) and subsequently washed with phosphate buffered saline (PBS) (Gibco), DMEM (pH 8, NaOH 0.01*M*) and DMEM. They were dried for 24 h at 25°C. Samples were seeded with 50 µL of a suspension of hDFs (1×10^6 cells mL^{-1}) and plated in 24-well cultures for 5 and 10 days, changing the medium every 24 h. For viability and cell attachment to the polymer with cells was washed with PBS and incubated with Hanks/Phenol medium with ethidium homodimer-1 (EthD-1) and calcein at 37°C for 45 min (Invitrogen). Then, the solution was removed, washed with PBS and analyzed in a microscope with fluorescent light (Axio Observer A1, Carl Zeiss).

Antimicrobial Activity Assay

Antimicrobial activity was evaluated on *Pseudomonas aeruginosa* (American Type Culture Collection strain 2783) grown in trypticase soy broth (TSB) at 36°C for 12 h to a concentration of 10^7 bacteria mL^{-1}. Samples were UV sterilized and washed with deionized water and TSB. Then, fresh was TSB added, inoculated with 10 µL of bacterial suspension and incubated at 36°C. Absorbance (595 nm) was recorded every 2 h during 24 h.

RESULTS AND DISCUSSION

Grafting of LA onto Ch

The proposed structures achieved in this work are depicted in Figure 1 for D and L lactic acid stereoisomer incorporation in the Ch repeat unit. The grafting by the C6 position on Ch by formation of an ester group is likely due to less steric hindrance. However, analytical evidences pointed out to the plausible formation of amide between LA and Ch up to some extent [Figure 1(C)]. The measured M_v for all grafted samples increased as compared with native Ch, as shown in Table I, and their solubility in acetic acid solution (0.1 *M*) decreased by three-fold. The latter might be ascribed to amide bond formation with lactyl units, which prevents Ch protonation, thereby reducing its solubility in acidic water. This assumption was corroborated by IR spectra, as shown in Figure 2, where a new

Table I. Properties of Unmodified and Grafted Ch

Material	M_v[1] ($\times 10^3$ $gmol^{-1}$)	Soluble matter in acetic acid 0.1*M* (%)[A]	DG (%)[B]	I_{CR} (%)	D_{ap}	Contact angle (°)[C]	Swelling (%)	Erosion (%)
Ch	959	93.7 ± 0.2^a	0	70.9	0.87	ND	ND	ND
A_{30}	977	34.6 ± 0.5^d	30.6 ± 0.2^c	61.4	0.71	73.5 ± 2.2^a	132.2 ± 28	26.6 ± 3.1
B_{55}	1026	48.1 ± 0.9^c	55 ± 0.2^b	69.6	0.44	94.1 ± 2.3^b	188 ± 18.4	25.4 ± 1.9
C_{75}	1115	53.6 ± 1.5^b	75.3 ± 0.1^a	70.1	0.76	94 ± 2.2^b	109.8 ± 17.1	13.4 ± 1.5

ND, non determined.
[A]As measured by ^{1}H NMR analyses. Data are shown as the average of five determinations with their corresponding standard deviation.
[B]Data are shown as the average of five determinations with their corresponding standard deviation.
[C]Data are shown as the average of 10 determinations with their corresponding standard deviation. Values in a column with the same letter are not significantly different at $P < 0.05$ determined by multiple comparisons of means by Tukey-Kramer test.

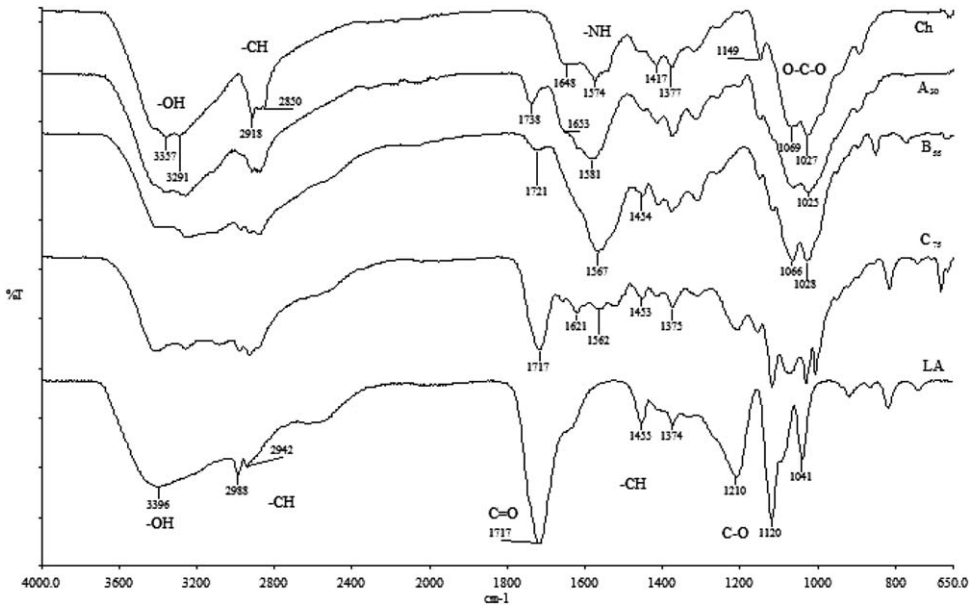

Figure 2. Infrared spectra of Ch, LA, and Ch-*g*-LA samples.

band observed at 1725 cm^{-1} in the A_{30} sample spectrum was assigned to the carbonyl groups in the grafted units. In addition, the absorption band at 1567 cm^{-1} from overlapping bands of amino groups and *N*-acetyl units evidenced that chitosan is linked to LA through amide bonds up to some extent.[6] Interestingly, the ATR-FTIR software showed 98% similarity for B_{55} and A_{30} spectra. ^{1}HNMR analyses corroborate the LA grafting with signals at displacements (δ) of 1.3–1.4 ppm assigned to the methyl of lactyl units. Methylene lactyl signals were not observed due to overlapping with the D_2O signal (Figure 3). The DG attained for each sample by integration of the characteristic signals in ^{1}H NMR spectra is shown in Table I. C_{75} displayed the highest incorporation having 1 : 3 ratio of lactyl : pyranosyl units, which correspond to a molar fraction of 75% of lactyl units. On the other hand, the %DG of B_{55} was 55% (1 : 5 lactyl : pyranosyl), whereas A_{30} show the lowest 30.63% grafted lactyl units (4 : 9 lactyl : pyranosyl). The TGA analyses displayed high thermal resistance for native Ch, as expected, with a weight loss of 25% at 300°C (see Supporting Information for TGA traces of native and *g*-Ch). The A_{30} with lowest lactyl grafting presents similar thermal profile, whereas that of B_{55} with the highest grafting has a moderate weight loss between 130 and 260°C owing to the less thermally stable ester moieties.[15]

Crystallinity of Ch and Ch- g-LA Samples

The LA was randomly grafted on the chitosan chain, which might decrease chain regularity thereby the observed reduction in I_{CR} and D_{ap} (see Supporting Information for X-ray diffraction analyses). Our semi-biologically obtained Ch displayed the typical X-ray diffraction pattern of an orthorhombic unit cell, with peaks about $2\theta = 10$ and 20°. The lactic acid fermentation-derived Ch possesses relatively high crystallinity and molecular weight as compared with chemically derived Chs, which might be of interest in biomedical applications, as reported elsewhere.[8] Nonetheless, the grafting decreased the intensity of the peak at $2\theta = 10°$ and in addition peak $2\theta = 20°$ widens due to the overlapping with peak $2\theta = 19°$, which was ascribed to lactyl crystallization because PLA homopolymer has 2θ values of 15, 17, and 19° with crystallization in a pseudo-orthorhombic unit cell.[8]

Mechanical Properties

The grafting onto Ch enhanced the mechanical properties in regard to the extension profiles and puncture testing in films, as it is shown in Table II. In the tension profile, C_{75} sample film was significantly ($P < 0.05$) the most flexible (Young's modulus = 48.2 MPa) followed by B_{55} (Young's modulus = 39.1 MPa) and the film from A_{30} sample (Young's modulus = 4.76 MPa). However, the latter displayed the highest elongation at break on extension (113%) followed by C_{75} (56.9%) and B_{55} (33.9%). As in the tension profile, C_{75} film sample was more elastic in puncture (Young's modulus 2.51 MPa), but the lowest elongation at break on pucture (16.3%), whereas B_{55} (Young's modulus 0.82 MPa) and A_{30} (Young's modulus 0.44 MPa)

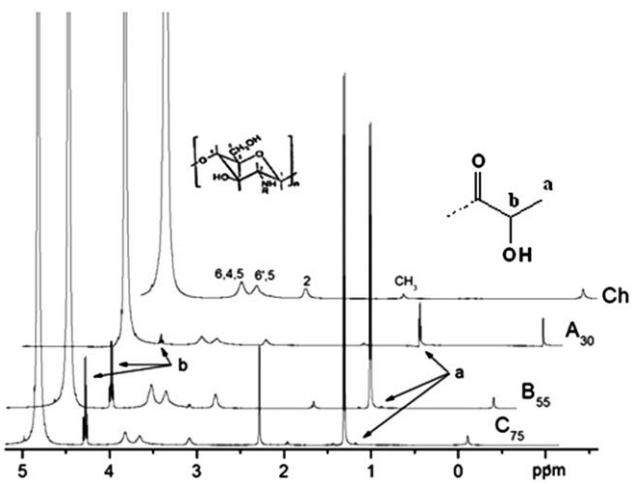

Figure 3. ^{1}H NMR spectra of Ch, LA, and Ch-*g*-LA samples.

Table II. Mechanical Properties Obtained from Tensile Strength to Extension and Puncture for Grafted Ch Samples

Sample	Young's modulus (MPa)	Tensile strength (MPa)	Percentage elongation at break (%)
Tension			
A_{30}	4.76 ± 0.30^{c}	4.67 ± 0.45^{b}	113 ± 11.20^{a}
B_{55}	39.10 ± 14.63^{b}	6.41 ± 1.37^{b}	33.9 ± 3.79^{c}
C_{75}	48.20 ± 9.52^{a}	15.99 ± 2.35^{a}	56.9 ± 7.43^{b}
Puncture			
A_{30}	0.44 ± 0.12^{b}	0.13 ± 0.04^{b}	23.6 ± 0.10^{a}
B_{55}	0.82 ± 0.38^{b}	0.23 ± 0.08^{b}	23.0 ± 1.11^{a}
C_{75}	2.51 ± 0.65^{a}	0.50 ± 0.08^{a}	16.3 ± 4.44^{b}

Data are shown as the average of five determinations with their corresponding standard deviation. Values in a column with the same letter are not significantly different at $P < 0.05$ determined by multiple comparisons of means by Tukey-Kramer test.

presented less elasticity than C_{75} but higher elongation at break on puncture ($B_{55} = 23\%$; $A_{30} = 23.6\%$). Therefore, it is inferred from the conducted mechanical tests that flexibility and elasticity were gained with the grafting as compared with our native Ch.

Swelling Capacities and Degradation Studies on the Synthetized Materials

The grafted samples behaved as hydrogels sensitive to pH changes, that is, when the pH decreases the concentration of charged ionic groups increase, creating an electrostatic repulsion between the adjacent amino ionized groups, leading to the relaxation of the chains and thus a greater swelling (see Supporting Information for swelling profiles during time). Contrarily, at increasing pH, the degree of ionization decreases and accordingly, the swelling of the films.[6,16] Interestingly, B_{55} film showed the highest swelling (Table I), which evidenced significantly ($P < 0.05$) the effect of the insertion of lactyl side units. This experimental evidence might be related to the relatively high swelling capacity reported for PLA.[7]

The degradation profiles of the grafted samples were determined under biological hDFs culture conditions (see Supporting Information for the graphical representation of the erosion through time in samples). Samples A_{30} and B_{55} were highly degraded, whereas the less eroded material was C_{75}. These results are in agreement with Xie et al.[17] study on Ch grafted with poly(lactide-*co*-glycolide)s where low polyester content displayed the fastest degradation rate. Degradation of the grafted samples might be related to their swelling capacity because the ability to absorb water promotes ester bond hydrolyses in the grafted chains, although in addition to that, degradation is a process, which takes days and also depends on molecular weights and crystallinity of copolymers.[15]

Contact Angle Studies

The wettability of our materials, which is an important parameter for promoting cell adhesion and growth, was determined by contact angle and the results are shown in Table I. Archana et al.[18] reported contact angles larger than 90° in hydrophobic-like surfaces, therefore according to that work our grafted materials can be described as relatively hydrophilic (Table I). However, B_{55} and C_{75} presented significantly higher contact angle than A_{30}, which is clearly related to LA incorporation. It is concluded that the higher lactyl units incorporated the higher hydrophobicity, in agreement to other reported works.[17]

Biocompatibility and Antimicrobial Activity Studies on the Synthesized Materials

Our viability tests are based on a direct method, in which the transformation of calcein to its polyanionic composite undergoes by ubiquitin, which produces a fluorescent green, and exclusion of the EthD-1 by plasma membrane occurs when cells were alive (Figure 4). Then, when the cell dies, EthD-1 enters the cell and produces a fluorescent red. Ch has been reported that promotes normal cell morphology, attachment, proliferation, and viability of living tissue cells.[19] Yao et al.[16] determined the hDFs growth on poly (chitosan-*g*-L-lactic acid) and Ch and similarly to the current study, the cell grew faster with Ch grafted copolymers than that with Ch. Then, regardless the DG, all the materials displayed viability and cell attachment, nonetheless, only A_{30} sample allowed hDFs elongation. This could be directly related to the lactyl-unit content in A_{30} because the degradation of lactyl units might generate acidic-side products that disturb cell growth. According to these and other reports, the degradation products of PLA can lead to local toxicity owing to acidosis with disturbance due to poor vascularization in the surrounding tissue on the site of implant.[16,19,20] Then, the absence of elongated hDFs observed in C_{75} and B_{55} samples is attributed to excessive acidification of the medium that generates decrease in the fibroblast growth. In addition, the surfaces of these films were relatively hydrophobic and therefore cells

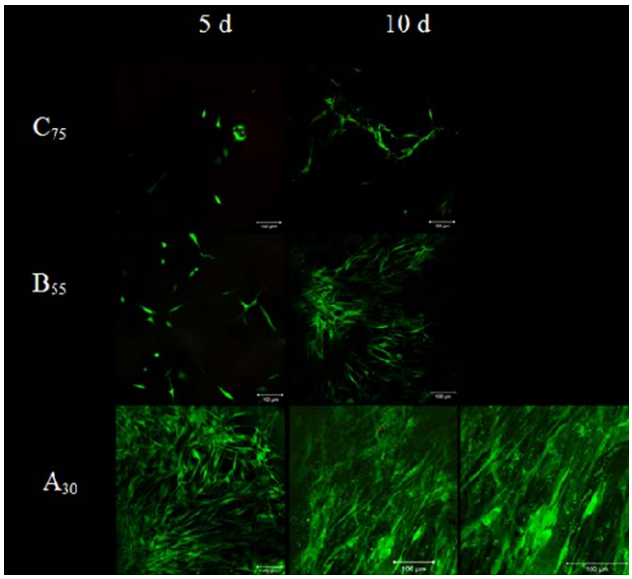

Figure 4. Fluorescence micrographs of the fibroblast cells onto copolymers with calcein assay. Live cells are visible as green (Calcein-AM), dead cells are observed as red (EthD-1). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

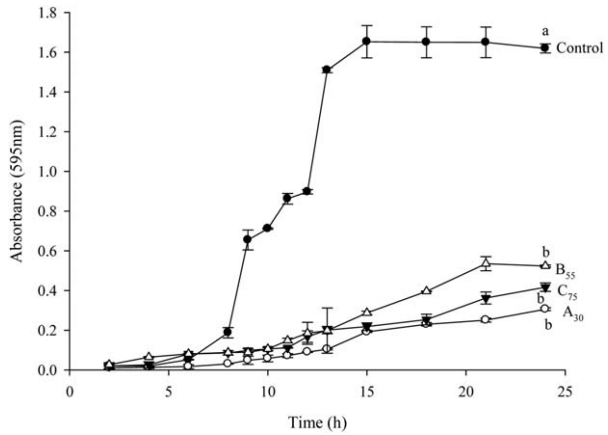

Figure 5. Time course of growth of *Pseudomonas aeruginosa* in TSB with the presence of grafted Ch samples. Materials with the same letter are not significantly different ($P < 0.05$) as determined by multiple comparisons of means by Tukey-Kramer test.

and media absorptions were relatively less favored on the surfaces of the synthetized materials. Our experimental evidences render the conclusion that high grafting of lactyl units onto our Ch chains might not be adequate for growth of hDFs despite of improved mechanical properties and reduced erosion.

Noteworthy, our semi-biological method to obtain the Ch,[8] which was used for grafting of lactyl units in this work, might present improved characteristics for cell adhesion over grafted materials using commercial Chs and further work is needed to address this evidence on a practical way.

In addition, microbiological tests were conducted to assess whether grafted polymers preserved the antimicrobial activity of Ch. The time course of *P. aeruginosa* growth in media with added materials is shown in Figure 5. The growth of the bacteria in control test presented a $\mu = 0.4811\ h^{-1}$, whereas *P. aeuruginosa* was inhibited in the presence of the grafted samples. A_{30} showed the highest inhibitory effect (87.59%), whereas for B_{55} was only 68.84% ($\mu = 0.1499\ h^{-1}$). Nonetheless, there were no significant differences ($P < 0.05$) among the three grafted samples regarding inhibition of this nosocomial pathogenic bacterium.

CONCLUSIONS

The synthetized material with the lowest lactyl units grafted onto Ch (DG 30.6%) allowed attachment and the highest viability and elongation of hDFs with high antimicrobial activity. All the grafted samples increase cell viability as compared with native Ch however, the increase in grafting played against fibroblast elongation. Accordingly, the enhanced grafting procedure with the presence of *p*-TSA catalyst give improved mechanical properties regardless the poorer biological response. The synthetized materials from our biotechnological chitosan present potential biomedical use and further work on application of these materials for TE is undergoing.

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