Research article

Poly(3-hydroxybutyrate) graft copolymer dense membranes for human mesenchymal stem cell growth

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ABSTRACT

Background: The use of novel materials as an artificial extracellular matrix for stem cell growth is a current strategy of increasing interest for regenerative medicine. Here, we prepare thermal-remolded membrane scaffolds from poly(3-hydroxybutyrate) grafted with 2-amino-ethyl methacrylate hydrochloride. However, it is unclear whether these membranes are useful for tissue engineering.
Results: The mechanical properties, tribology, and morphology of the dense membranes were assessed. The results show that tensile strain at break and roughness of the compressed membrane decrease with increasing graft degree. Moreover, graft copolymer membranes showed lower resistance to scratching, greater degree of swelling and higher brittleness than un-grafted P(3HB) films. Thus, it effectively supports the growth of dermal fibroblast, as demonstrated by epifluorescence microscopy.
Conclusions: It is concluded that the developed membrane can be properly used in is the restoration of skin tissue.
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1. Introduction

Advances in the practical applications of poly(3-hydroxybutyrate) (P(3HB)) as biocompatible [1] and biodegradable [2] polyester have been hampered by its low mechanical strength and hydrophobicity [3]. To overcome these drawbacks, several alternative techniques have been developed [4], including, blending and grafting [5,6]. Thus, P(3HB) has been blended with poly(l-lactic acid) and 3D Fourier transform infrared (FT-IR) spectroscopic imaging was used to study the phase structures of the P(3HB) + PLA blends [7]. Grafting of P(3HB) films with poly(acrylic acid) by plasma-initiated polymerization has also been reported [8].

Gamma radiation has attracted interest as a suitable technique that has also been used to modify P(3HB). Mitomo et al. [9] applied gamma-radiation-graft polymerization for grafting 2-hydroxyethyl methacrylate and methyl methacrylate onto P(3HB-co-HV) [10]. Luk et al. [11] have reported the grafting of AEMA on the P(3HB-co-HV) films.

Alternative high-energy sources, such as plasma [12] and UV irradiation [13], have been used to graft moieties onto polyhydroxyalkanoates. Previously, we have modified the surface of P(3HB-co-HV) with plasma and N-hydroxyethyl-acrylamide to prepare an amine functionalized scaffold [14]. However, these techniques are limited since they modify only the surface of the polymers, while the advantage of gamma radiation is that it affects the P(3HB) bulk properties [15,16,17].

Practical applications of modified and unmodified P(3HB) scaffolds have previously been discussed [18]. Various routes of fabrication and characterization of scaffolds have been undertaken such as: nano-sized hydroxyapatite (HA)/poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBV) composite [19,20]; preparation of three-dimensional scaffolds of P(3HB)/poly(3-hydroxybutyrate-co-hydroxyhexanoate) (PHBHHx) to produce neocartilage with stem cells [21] and for tendon tissue engineering [22]; electrospun fiber mats [23]; preparation of P(3HB)

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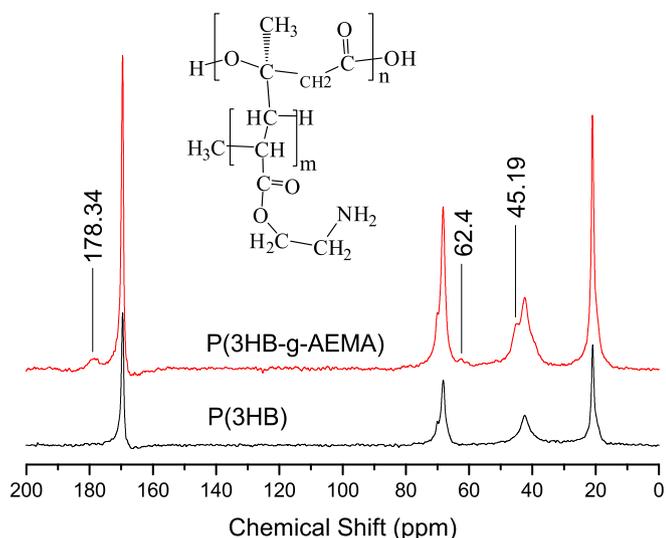
Fig. 1. ^{13}C-CPMAS-NMR spectrum of P(3HB) and P(3HB-g-AEMA). (P1M1S3/ 14% of degree of grafting).

foams by the solvent casting + particulate leaching techniques [24]; and the use of pore forming agents [25].

Sombatmankhong et al. [23] reported on the fabrication of ultrafine fiber mats consisting of P(3HB), poly (3-hydroxybutyrate-co-2-hydroxyvalerate) (PHBV), and 50/50 w/w blends for scaffold applications. All fibrous scaffolds exhibited much better support for cell attachment and proliferation than solution-cast film scaffolds. The results of Sombatmankhong et al. [23] are in line with our idea of using copolymers containing P(3HB) for scaffold development. However, solution cast-film and thermal remolded membranes are certainly worthy of further exploration for biological applications.

In an earlier work, we prepared P(3HB-g-AEMA) thermal-remolded membranes, evaluated their crystallinity and studied them by vibrational spectroscopy [26]. However, it is not yet known whether they can be directly used as scaffolds for tissue engineering. Here, we aimed to characterize the P(3HB-g-AEMA) using new methods to examine their physical–chemical and structure–function properties. To the best of our knowledge, this is the first time that the biological functionality of this type of amine-functionalized polyhydroxyalkanoates (dense membrane) is evaluated. Possibility of using such materials as scaffolds for human mesenchymal stem cell growth was explored.

2. Experimental procedures

2.1. Materials

P(3HB) (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in chloroform and purified before use by precipitation in acetone. The solvents (99.9%, Sigma-Aldrich), ethanol (S1) hexane (S2) ethyl acetate (S3), chloroform (S4), acetone (S5) and acetic acid (S6),
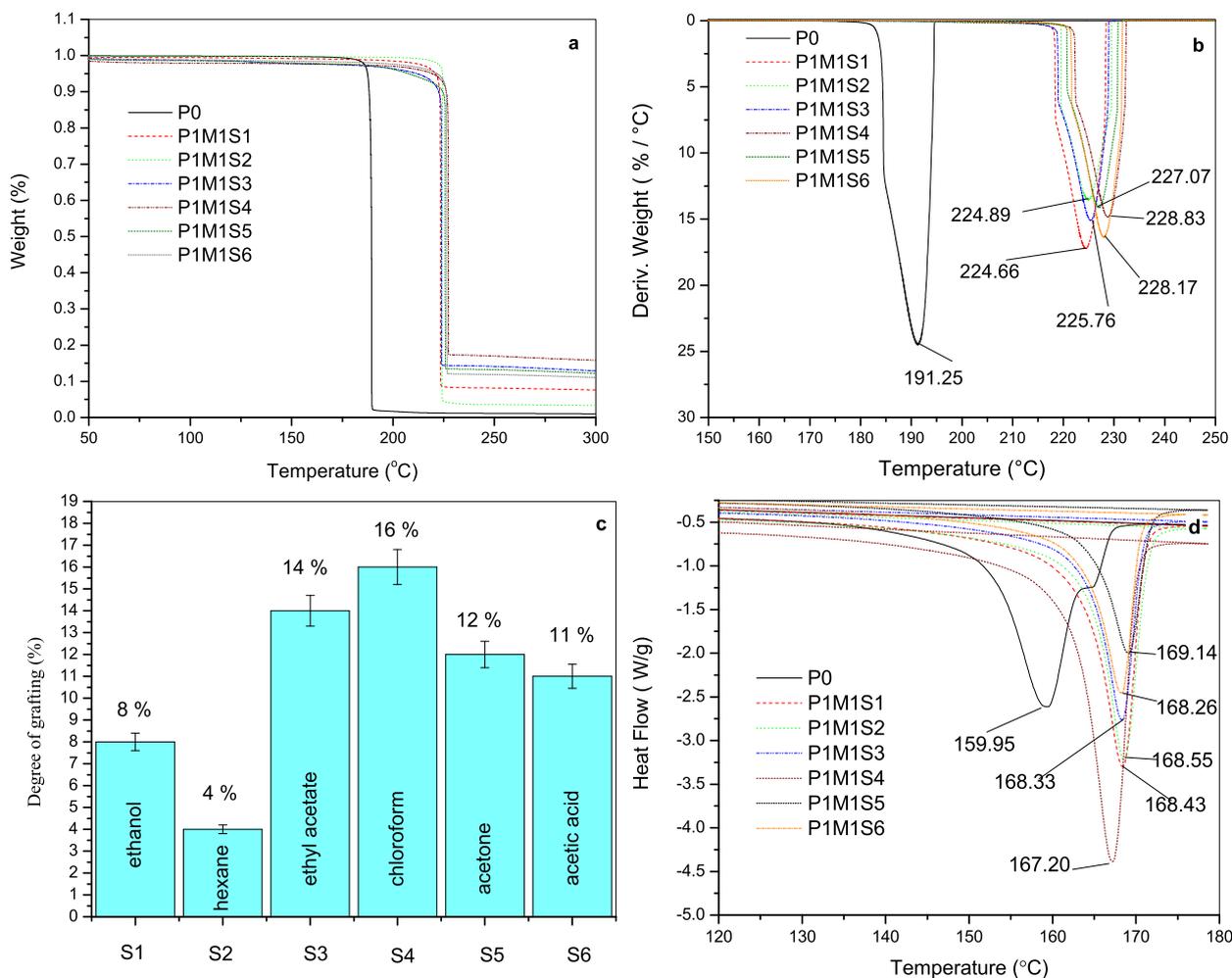
Fig. 2. (a) Top left: THERMOGRAMS (TGA) of P(3HB) and grafted polymers; (b) top right: derivative thermogravimetric curves (DTGA) of P(3HB) and grafted polymers; (c) bottom left: degree of grafting obtained using different types of solvents; (d) bottom right: DSC of P(3HB) and grafted polymers.

Table 1
Some thermodynamic parameters from the P(3HB) and P(3HB-g-AEMA) calorimetry.

Sample	W/ (%) (TGA)	ΔH_m (J/g)	T_m/(°C)	T_{onset}/(°C)
P(3HB)	0	88	159	186
P1M2S1	8	80	168	220
P1M2S2	4	83	168	223
P1M2S3	14	74	168	221
P1M2S4	16	68	167	225
P1M2S5	12	66	169	224
P1M2S6	11	68	168	221

were used as received. The monomer, 2-amino ethyl methacrylate, in its hydrochloride form (Sigma-Aldrich, Germany) was also used as received without further purification.

2.2. Synthesis of P(3HB-g-AEMA) derivative and thermal remolded membranes

The syntheses of the AEMA functionalized P(3HB) were carried out through simultaneous irradiation method as reported before [26]. ^{60}Co-γ-radiation in air (Gamma Beam 651 PT, Nordion International), was the source of gamma rays at a dose of approximately 11.9 kGy and 2.0 kGy/h dose rate, measured by a Fricke dosimeter. For the syntheses procedures, glass ampoules were properly sealed. For the graft reaction, 100 mg of monomer was added to 350 mg of P(3HB) and mixed with two mL of solvent. The grafted P(3HB) with AEMA

monomer was purified by Soxhlet extraction. Acetone was used for 72 h in a Sohxlet to ensure the purification of the copolymer and the elimination of AEMA and PAEMA residues. The products obtained were finally dried at about 50°C under vacuum conditions. Each grafting was repeated three times. We shall use the following symbols: P0 refers to untreated poly(3-hydroxybutyrate), while P1 refers to P(3HB) when it has reacted, or it is part of a modified molecule; M1 is the AEMA monomer; for instance, P1M1S2 is the graft copolymer of P(3HB) with AEMA in hexane as solvent.

The route of dense membrane scaffolds preparation was by solvent casting from CHCl3 and a hot press at 170°C, at a pressure of approximately 4×105 kg/m^2 for 20 min. Scaffolds of about 1.5 cm^2 and with 0.4 mm thickness were obtained.

2.3. Nuclear magnetic resonance of P(3HB-g-AEMA) powder

The solid-state ^{13}C-CPMAS-NMR of P(3HB-g-AEMA) was recorded at 75 MHz on a Bruker Avance II 300 MHz NMR spectrometer. About 150 mg of each sample was introduced into a 7.5 mm rotor. The contact time was established at 2 ms.

2.4. Thermophysical properties of P(3HB-g-AEMA) powder

The thermal analysis was carried out with a Thermogravimetric (TGA) Analyzer and Differential Scanning Calorimeter STA F 449 Jupiter from Netzch. TGA of the samples (14 mg) was performed
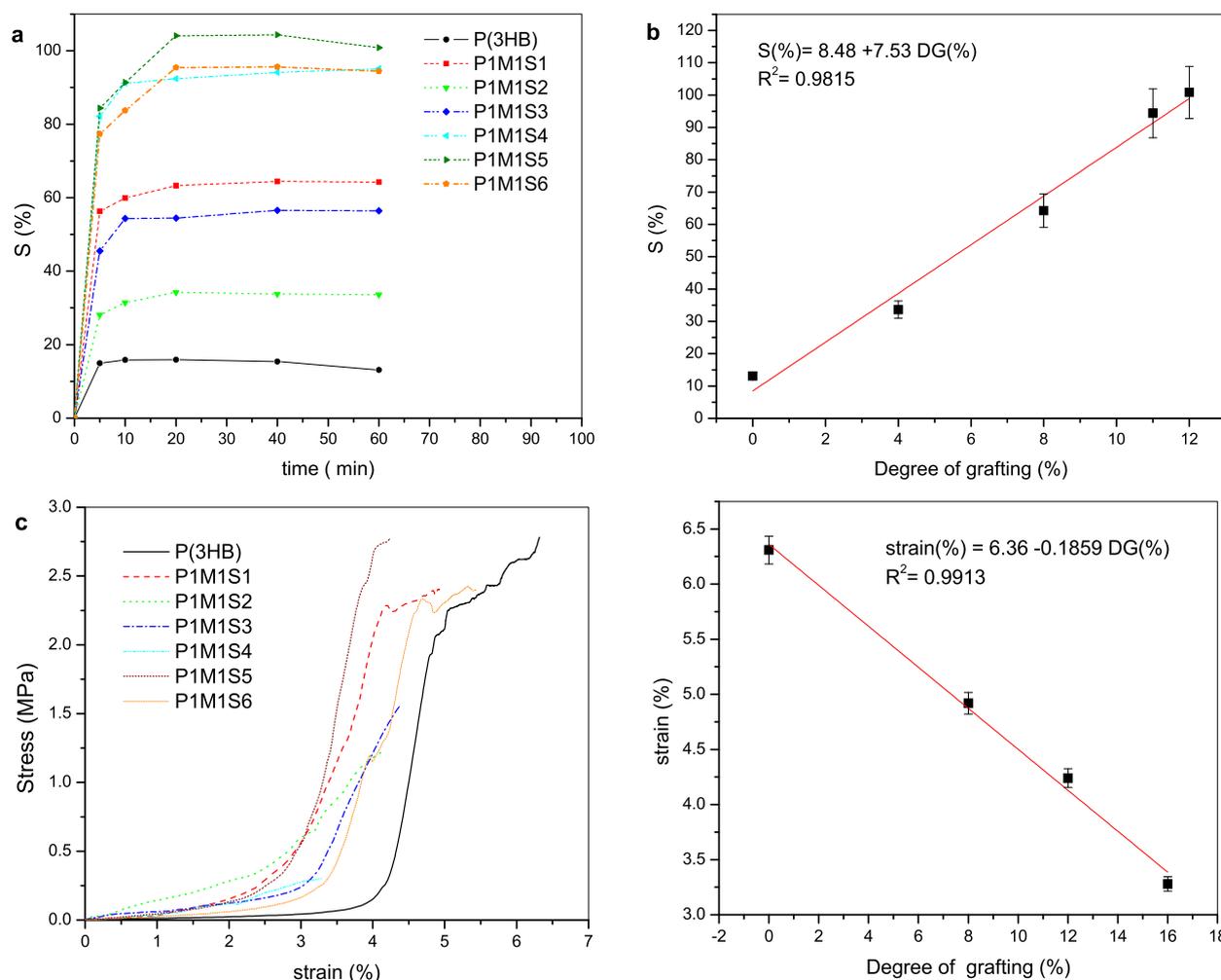
Fig. 3. (a) Swelling percentage S/ (%) versus time; (b) S/ (%) as a function of W/ (%) of grafted polymers. (c) stress vs. strain curves of P(3HB) and grafted copolymers obtained in different solvents; (d) strain (%) as a function of the degree of grafting (W (%)).

Table 2
Mechanical properties of P (3HB) and P(3HB-g-AEMA) grafted polymers.

Sample	E/(MPa)	σ_b(MPa)	ε_b (%)	A toughness
P(3HB)	1.10 ± 0.1	8.60 ± 0.13	6.31 ± 0.21	0.68 ± 0.04
P1M1S1	3.75 ± 0.1	9.06 ± 0.13	4.92 ± 0.21	0.64 ± 0.04
P1M1S2	19.6 ± 0.1	2.52 ± 0.13	4.14 ± 0.21	0.49 ± 0.04
P1M1S3	23.9 ± 0.1	3.57 ± 0.13	4.37 ± 0.21	0.60 ± 0.04
P1M1S4	7.05 ± 0.1	0.98 ± 0.13	3.28 ± 0.21	1.14 ± 0.04
P1M1S5	4.83 ± 0.1	4.38 ± 0.13	4.24 ± 0.21	1.21 ± 0.04
P1M1S6	3.96 ± 0.1	4.80 ± 0.13	5.41 ± 0.21	0.90 ± 0.04

under a nitrogen atmosphere at a heating rate of 10°C/min from 40 to 600°C. Differential scanning calorimetry (DSC) was carried out as follows: heating of the samples from −40°C to about +170°C (first heating scan) at a rate of 10°C/min, then keeping the samples at 170°C for a few minutes. Once the thermal history was thus erased, the samples were first cooled to −40°C at 10°C/min and finally heated from −40°C to 170°C at the same heating rate (second heating scan). The enthalpy of melting and the melting temperature were resolved from the endothermic peak in the second run.

2.5. Dense membrane's characterization

2.5.1. Swelling studies

The water sorption capacity of P(3HB-g-AEMA) and P(3HB) thermal-remolded membranes scaffolds was carried out by immersing the polymers in water at ambient temperature for 2 h as described in the ASTM D570 standard. The degree of swelling was measured by the formula: $S(\%) = \dfrac{m - mo}{mo}\,100$, which provides the increment of the mass with respect to the initial mass of the sample at different time intervals until a constant weight is achieved.

2.5.2. Mechanical assessment

The tensile testing of thermal-remolded membranes was carried out using a Zwick/ROELLZ005 universal testing machine at a speed of 0.01 mm/min following the ASTM D882 standard.

2.5.3. Scratch resistance

The tests were carried out using a CSM Micro-Scratch Tester (MST), from Neuchatel, Switzerland. Single scratches were performed under the following conditions: progressive load from 0.03 N to 10 N; scratch length 5 mm; 5.33 mm/min scratch speed at the room temperature (24°C). A conical diamond indenter was used in all the tests with the diameter of 200 μm and the cone angle 120°.The instantaneous penetration depth R_p and the residual (healing) depth R_h are thus obtained as a function of the applied load. The technique has been explained in review article [27].

2.5.4. Morphology study of the membranes

The morphologies of thermal remolded membrane scaffolds were observed in the tapping mode with a JEOL JSPM 5200 AFM equipped with a silicon nitride cantilever (Micromash). The peak frequency was at 349 kHz, and the quality factor was 1031,003. The scanning speed and loop gain were varied depending on the conditions of each sample.

2.5.5. Cell viability onto thermal membranes

A preliminary biological study was performed. Thermal membrane samples of 5 × 5 mm^2 were cut and sterilized with UV light prior to fibroblasts seeding. Primary dermal fibroblasts were isolated from skin remnants of esthetic surgeries from healthy patients who responded to an Informed Consent. Fibroblasts were isolated as previously reported [28]. Briefly, skin biopsies were mechanically fragmented and incubated in dispase II; then, epidermis was discarded, and dermis was digested in Type I collagenase. Fibroblasts were recovered by centrifugation, re-suspended in supplemented (1% penicillin–streptomycin and 10% fetal bovine serum; GIBCO) DMEM-F12 (GIBCO) and seeded in culture flasks. Fibroblasts were expanded until passage 3, when they were detached with tripsin (0.25%, GIBCO), counted, seeded in drops (15,000 cells in 10 μL) onto the membranes at a final concentration of 120,000 cells/cm^2, and incubated during 1 h at 37°C and 5% CO2; experiments were performed by triplicate. After that, samples were covered with supplemented DMEM-F12 and further incubated for 24 h. Finally, cell viability was assessed using the Live/Dead™ viability/cytotoxicity kit for mammalian cells (Thermo), following kit's manufacturer technical guidelines. Briefly, membranes were incubated
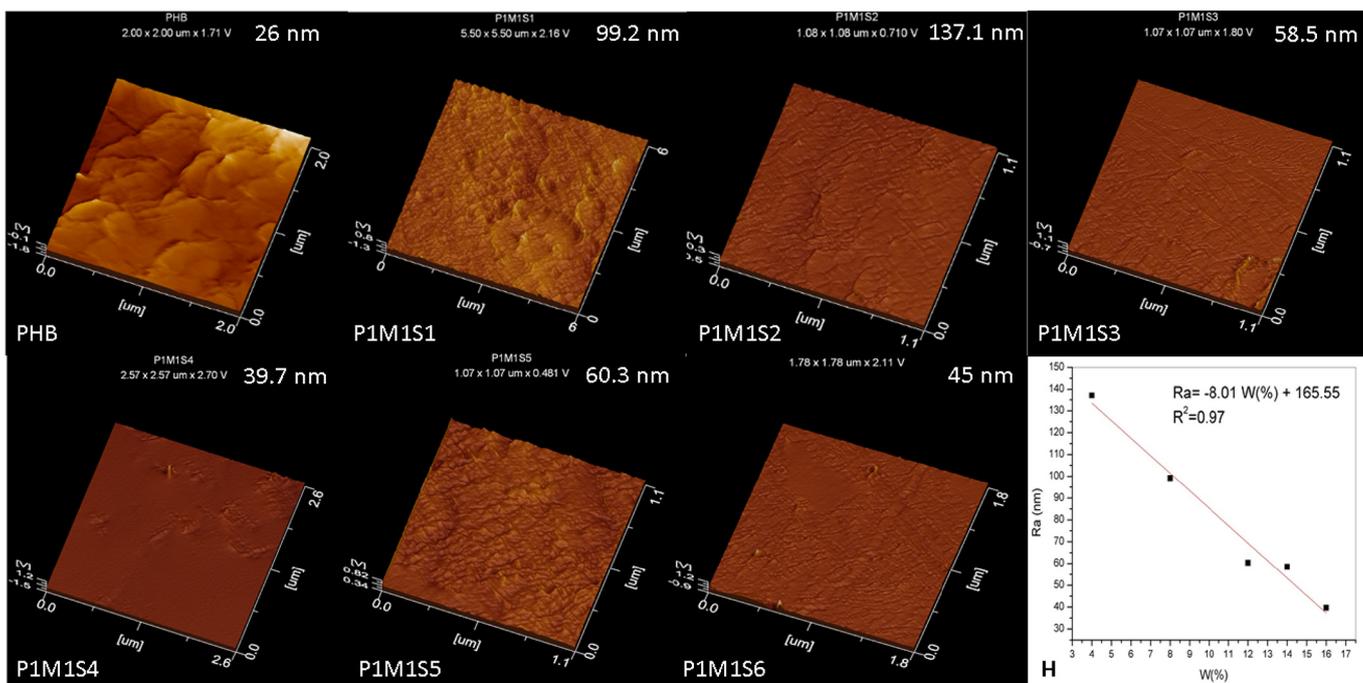
Fig. 4. Two-dimensional AFM image thermal remolded membrane scaffolds and relationship of their roughness with the degree of grafting.

with 1 μM calcein AM and 2 μM ethidium homodimer solution (diluted in Hank's medium) during 1 h and immediately visualized by fluorescence microscopy (Carl Zeiss). Images were processed with AxioVision software®, live and dead cells were counted and percentage of each condition (live/dead) over total number of cells was calculated.

3. Results and discussion

3.1. Solid-state nuclear magnetic resonance of P(3HB-g-AEMA) powder

Fig. 1 shows the solid-state ^{13}C-CPMAS-NMR of P(3HB-g-AEMA) (P1M1S3) with a grafting structure proposal. The NMR was carried out to detect evidence of the grafting of AEMA onto P(3HB). Three novel signals were found in the spectrum, at 45.2, 62.4 and 178.3 ppm; they may be attributed to new methylene, quaternary substituted carbon, and carbonyl groups respectively. The rest of the signals were characteristic of the P(3HB) sample: (CH$_3$ (21 ppm), CH$_2$ (42 ppm), CH (68 ppm), and C=O (169 ppm) [29,30].

3.2. Thermophysical properties of P(3HB-g-AEMA) powder

TGA and DTGA curves for P(3HB) and P(3HB-g-AEMA)., obtained in different solvents, are shown in Fig. 2a, b. The solvent is one of the three obvious factors that change the degree of grafting, the other two factors are the dose rate and monomer concentration, which remained constant [31]. As can be observed, the decomposition temperature of the grafted copolymer is different from the control at 224–228°C. The differences in the decomposition temperature between the grafted materials are small. Furthermore, both P(3HB) and the copolymers have smooth weight loss curves. However, P(3HB-g-AEMA). shows two steps in the decomposition curves instead of one for P(3HB).

The degree of grafting (W(%)) was evaluated as defined by Mitomo et al. [32] as the ratio of complete weight loss to weight loss obtained at the intersection of the steepest tangent between the first step and the plateau, preceding the second-step drop. Thus, the value was obtained from the increase of mass after grafting divided by the initial mass of the poly(3-hydroxybutyrate). The degrees of grafting obtained from
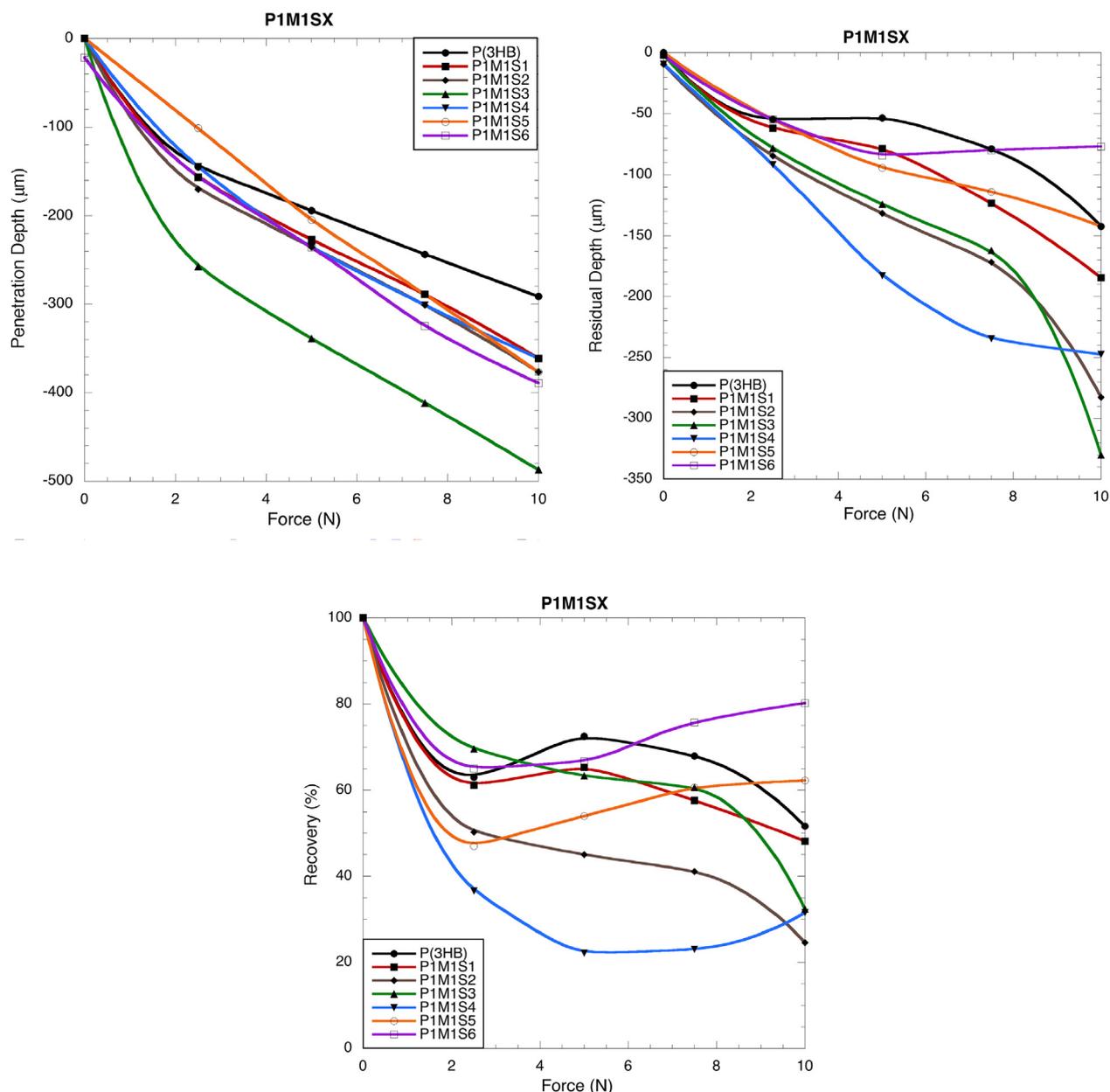
Fig. 5. Single scratch penetration depths, residual depths and viscoelastic recovery of neat P(3HB) and P(3HB-g-AEMA) thermally remolded membrane scaffolds as function of the applied force.

the TGA curves are as follows: the lowest degree of grafting value was obtained from hexane (4%), and increasing values of 8%, 11%, 12%, 14% and 16% were acquired from ethanol, acetic acid, acetone, ethyl acetate and chloroform, respectively (Fig. 2c). The highest value from chloroform is consistent with the results obtained for the radiation induced grafting of acrylamide onto P(3HB) [33]. It is of note that the degree of grafting (DG) obtained by mass balance in our previous work with the eq. $DG(\%) = \frac{(m_g - m_o)}{m_o} * 100$, where $m_o(g)$ is the initial weight of the P(3HB) and $m_g(g)$ is the P(3HB-g-AEMA). Weight is in agreement with (W (%)) results [26].

We see in Table 1 and Fig. 2b that the grafted polymer is more stable than P0 (pristine P(3HB)); the onset of degradation increased from 191°C to 220–225°C. The second heating runs of DSC of grafted and control P(3HB) are shown in Fig. 2d; some thermodynamics parameters derived from DSC are also listed in Table 1. Grafting moves upwards the melting point $T_m/(°C)$ by about 10°C, while the enthalpy of melting (ΔH_m (J/g)) decreases with respect to P(3HB). The result obtained is consistent with the appearance of one peak in the DSC analysis (Fig. 2d). This also confirms that the AEMA was graft polymerized onto the poly(3-hydroxybutyrate) [11,34,35].

3.3. Swelling studies and mechanical properties of thermal-remolded membranes

Fig. 3a shows the swelling percentage S (%) versus time for the thermal-remolded membranes. We see that an increasing degree of grafting results in an increase of swelling (Fig. 3b). A relationship appears to be linear, with a correlation coefficient $R^2 = 0.982$ (R^2 is equal to unity for a perfect fit). Thus, we find that the thermally remolded P(3HB-g-AEMA). membrane scaffolds are more hydrophilic than those made from P(3HB) and are also thermally more stable, a desired result for tissue engineering applications.

As can be seen from Fig. 3c, d, the tensile testing was performed on the control and grafted P(3HB) thermal-remolded membrane scaffolds. The stress at break ($\sigma_b/(MPa)$), elongation at break ($\varepsilon_b/(\%)$), the tensile toughness defined [36] as the area under the stress vs. strain curve (A)

and the Young's Modulus (E/(MPa)) are listed in Table 2. The stress σ vs. strain ε curves are not linear; a low strain rate was applied because the samples were brittle. As shown in Table 2 and Fig. 3d, the increase of the degree of grafting involves a linear decrease in the strain percentage, in agreement with preceding work [36,37,38].

3.4. Analysis of the roughness of thermal-remolded membranes

We now consider roughness. AFM images in Fig. 4 reveal that the roughness of thermally remolded membrane scaffolds is higher than that of pristine P(3HB). An increase of the degree of grafting leads to a decrease of the roughness in grafted samples; see the insert in the Fig. 4. So at first sight, the porosity of the films seems inadequate for the cell seeding [39].

3.5. Scratch resistance of thermal-remolded membranes

In scratch resistance determination we observe the viscoelastic recovery f defined [25,26] as:

$$f = [(R_p - R_h) * 100\%] / R_p \qquad \text{[Equation 1]}$$

Fig. 5 shows the results. We chose 0.03 N to 10.0 N as the loading range because of the thickness (<0.5 mm) of the samples. If we consider the results for 10 N, the shallowest recovery depth R_h is seen for P1M1S6, followed by P(3HB). P1M1S6 has also the highest viscoelastic recovery at 10 N. We recall that f decreases with increasing brittleness B and there is for polymers an equation relating the two quantities [38,39,40]. The recovery values are on the low side, in agreement with the elongation at break ε_b values seen above.

3.6. Thermal membranes show high citocompability

After 24 h of culture of fibroblasts onto thermal membranes, the analysis of cell viability showed that most cells (>99%) were alive, positive for calcein, with less than 1% of dead cells, EthD-1 positive; Fig. 6. It is also important to emphasize that cells showed the expected
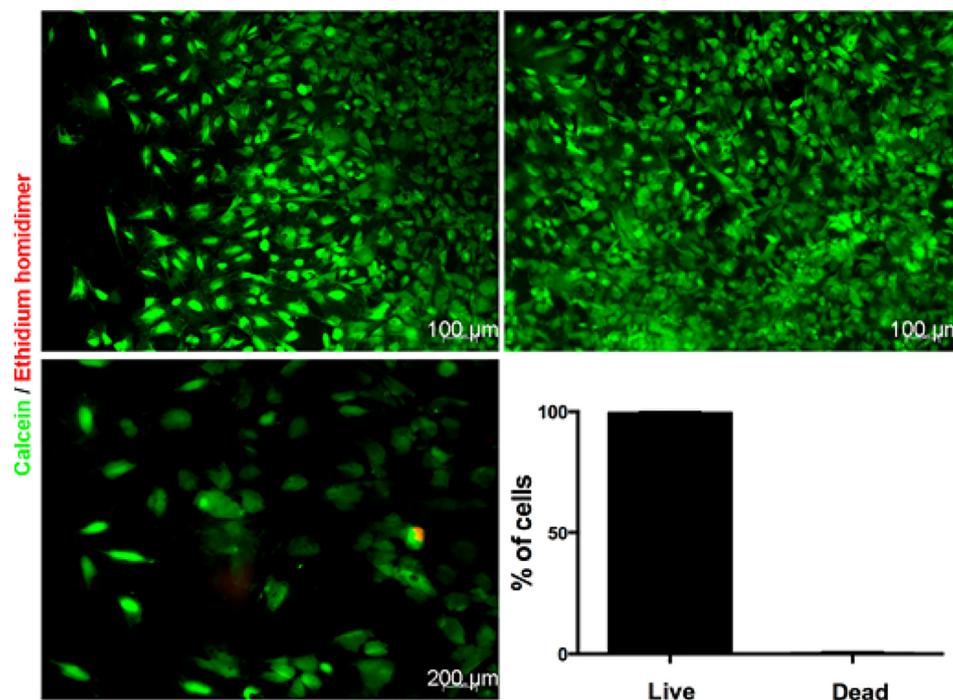
Fig. 6. Thermal membranes support dermal fibroblast culture. Figure shows representative fluorescence micrographs of cell viability assay after 24 h of fibroblasts culture onto thermal membranes; live cells are stained with calcein (green) and dead cells are stained with EthD-1 (red). Graph shows percentage of live and dead cells over total number of cells on thermal membranes.

fusiform morphology of adhered fibroblasts upon culture onto the thermal membranes, suggesting that cells were properly adhered to the membranes. These two results (high cell viability percentage and proper morphology) suggest that thermal membranes are biocompatible materials with potential to be used as cells carriers for treatment of skin lesions.

Hereafter, we mentioned that two drawbacks in the use of poly(3-hydroxybutyrate) for tissue engineering applications are the lack of functional groups as well as its hydrophobic nature. Here we addressed this issue by preparing the AEMA functionalized membrane. As can be seen, the grafting did not decrease the viability of the material compared with un-grafted P(3HB) [40]. An additional advantage would be the increase of capability of protein linkage by control of the distribution of amine groups in the surface [11].

4. Conclusion

In this work, we demonstrated that 2-amino ethyl methacrylate hydrochloride can be successfully grafted onto poly(3-hydroxybutyrate) using supplementary characterization method. New evidence for grafting was obtained by ^{13}C-CPMAS-NMR and TGA/DSC. Different degrees of grafting were obtained by varying the solvent; the best-swelling solvents, ethyl acetate and chloroform, provide the highest degree of grafting (P1M1S3 & P1M1S4). The products so obtained are more thermally stable than P(3HB). Thermal-remolded membranes were obtained from the P(3HB) and grafted biopolymer. Increasing degree of grafting results in an increase of swelling and lowering of the strain at break and roughness of the dense membranes. The scratch resistance experiments verified that the membranes are very brittle. The scaffolds appropriately support culture of dermal fibroblasts. To conclude, it is shown that the proposed membranes are useful for skin tissue engineering and skin wound treatment.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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