Membranes of chitosan grafted onto poly(3-hydroxybutyrate): new insights into their applicability as scaffolds

F. Quintanilla[1], J.R. Rodríguez[2], S. Vargas[2], S. G. Solís[3], R. Olayo[4], N. Hnatchuk[5], W. Brostow[5], Ikang Chen[5] and M. González[2]* (iD)

Chitosan was grafted onto poly(3-hydroxybutyrate) (P(3HB)) as in our earlier work by using gamma radiation-induced polymerization reaction. We now provide structural and surface characteristics of the graft copolymer in view of potential applications. The modified P(3HB) was characterized by ^{1}H/solid-state ^{13}C CP-MAS nuclear magnetic resonance, Fourier transform infrared spectroscopy, thermogravimetric analysis, atomic force microscopy, contact angles and scratch resistance determination. Copolymer surfaces resemble those of neat P(3HB) and are unsuitable for membranes. However, a novel composite based on grafting copolymer and polyurethane was developed. It displays good properties for the preparation of 3D-scaffolds with potential uses in tissue engineering.

Keywords: Poly(3-hydroxybutyrate), Chitosan, Graft polymerization, Gamma radiation, Tribology, Composite

Introduction

The range of applications of biodegradable materials, such as polyesters and polysaccharides, can be extended by their physical (i.e. blends) or chemical (i.e. graft copolymerization) modifications.[1–3] These materials have been studied for many decades due to their wide use in biomedical applications as well as in search for new pharmaceutical products.[4, 5] From the polyester family, poly(3-hydroxybutyrate) (P(3HB)) seems to be the most studied polyhydroxyalkanoate due to its biodegradability and biocompatibility properties,[4] while chitosan (CS) has similar properties as a polysaccharide family member.[5]

Both P(3HB) and CS have been chemically modified so as to obtain novel polymeric materials. The graft polymerization has been an alternative approach, with good results, to develop new bioderivatives.[6, 7]

Graft polymerization of P(3HB) using high-energy radiation, has been extensively studied using UV,[8–10] plasma[11, 12] and gamma ray sources.[12] In particular, gamma radiation makes possible chemical grafting reaction through the complete material and not only on the surface.[10, 13, 14]

Earlier, we obtained the material chitosan (CS) grafted onto P(3HB), hereafter called P(3HB-g-CS), using gamma radiation-induced graft polymerization.[15] However, a study on the structure and functional properties of the grafting copolymer is still needed in view of possible applications.

We describe here a novel method of characterization of the P(3HB-g-CS) and its structure, so as to propose suitable applications in tissue engineering.

A different type of graft copolymer of P(3HB) and CS has been synthesized previously.[16] Hu et al. used ozone to insert acrylic acid and then grafted chitosan onto the P(3HB-g-AAc) copolymer. That material showed higher antibacterial activity and lower biodegradability than P(3HB). Radiation-induced P(3HB-g-CS) has been created solely by us, its applicability not yet reported.

Experimental

Materials

P(3HB) was obtained from Sigma Aldrich Co. It was dissolved in chloroform and purified by precipitation from hexane. The solvents, also from Sigma Aldrich Co. were: hexane, ethyl acetate, chloroform, acetone, ethanol and acetic acid; they were used as received and are named: S2, S3, S4, S5, S1 and S6, respectively. The chitosan sample (CS), supplied by Sigma Aldrich Co, was used without further purification.

Methods

Synthesis

The synthesis of P(3HB)-g-CS was performed with a simultaneous irradiation method so that both polymers were subjected to the same radiation level. [60]Co gamma ray source

[1]Department of pharmaco chemical, University of the Valley of Mexico (UVM), Boulevard Villas del Mesón 1000, Juriquilla, Santiago de Querétaro, Querétaro 76320, Mexico

[2]Department of Molecular Engineering of Materials, Center of Applied Physics and Advanced Technology, National Autonomous University of Mexico (CFATA-UNAM), Boulevard Juriquilla 3001, Santiago de Querétaro, Querétaro 76320, Mexico

[3]Department of analytical chemistry, Research Center for Applied Chemistry (CIQA), Blvd. Enrique Reyna Hermosillo # 140 Col, San José de los Cerritos Saltillo Coahuila 25294, Mexico

[4]Department of Physics, Autonomous Metropolitan University (UAM), Av. San Rafael Atlixco N° 186, Col. Vicentina, Del. Iztapalapa, México, DF 09340, Mexico

[5]Laboratory of Advanced Polymers & Optimized Materials (LAPOM), Department of Materials Science and Engineering and Department of Physics, University of North Texas, 3940 North Elm Street, Denton, TX 76207, USA

*Corresponding author, email mikegcu@gmail.com, mikegcu@fata.unam.mx

was used at a dose of about 12 kGy, with 2 kGy/h dose rate measured with a Fricke dosimeter. The synthesis procedure consists of the use of glass ampoules suitably sealed containing a mixture of both polymers and the solvent. Graft reaction was performed using 100 mg of each polymer mixed with 1.5 mL of the solvent. The copolymer P(3HB) grafted with CS was washed with trichloromethane to eliminate the ungrafted P(3HB) and then purified by Soxhlet extraction using acetone for several days. The P(3HB)-g-CS obtained was finally dried at about 45 °C in an oven until a constant weight was reached.

The thermal remoulded membranes were obtained in a template (Teflon). The grafting copolymer samples were heated to 180 °C at a pressure of approximately 3×10^5 kg/m^2 for 15 min. Afterwards, the membranes were cut into pieces of about 1.5 cm^2 in size. The membranes' thickness was around 1.2 mm.

The grafting copolymer composite (hereafter called GCC), was prepared by the addition and mixing of 0.1 g of P(3HB)-g-CS powder, sodium acid carbonate (NaHCO$_3$) and polyurethane (polyester-hydroxylated resin/polyisocyanate 1/4 (v/v)). The Teflon mould was filled with the as-obtained dough. GCC scaffolds of approximately 1.7 mm thickness and 1.2 cm^2 area were used. Finally, the GCC scaffolds were soxhlet extracted with distilled water for two days in order to eliminate the pore former.

We shall henceforth use the symbols as follows: P0 refers to P(3HB) control sample, while P1 is the polymer after it has reacted. Similarly, QS is the chitosan blank and Q1 represents grafted CS; for instance, P1Q1S3 is the graft copolymer of P(3HB) with CS in ethyl acetate media, etc. The experiments were repeated three times to guarantee reproducibility.

Nuclear magnetic resonance

The proton nuclear magnetic resonance (^{1}H-NMR) spectrum was acquired on a Bruker Avance III HD 500 MHz (500 MHz, standard 5 mm quartz probe). CF$_3$COOD was used as the solvent. Solid-state ^{13}C CPMAS NMR of P(HB-g-CS) was recorded at 75 MHz on a Bruker Avance II 300 MHz NMR Spectrometer. About 150 mg of the samples was introduced into a 7.5 mm rotor. The contact time was established at 2 ms.

Thermogravimetric analysis

We have used a STA F 449 Jupiter model Netzsch TG Analyzer. 10 mg samples were analyse the under N$_2$ atmosphere at 10 °C/min heating rate; the temperature range covered was from ambient to 600 °C.

FT-IR measurements

The FTIR spectra of P(3HB-g-CS) were obtained in a Bruker Vector 33 spectrometer with a resolution of 4 cm^{-1} in the transmittance mode at room temperature. The range from 4000 to 700 cm^{-1} was examined.

Degree of grafting

The degree of grafting DG/(%) was calculated with the thermogravimetric analysis (TGA) technique using the method previously described by Mitomo et al. (1996) where the mass increase of the graft copolymer is calculated[17] as

$$\text{DG}/(\%) = [(m_{\text{P(3HB)-g-CS}} - m_{\text{P(3HB)}})/m_{\text{P(3HB)}}] \times 100 \quad (1)$$

Scratch resistance determination

Single scratch tests were conducted on a microscratch tester (CSM Instruments, Peseux, Switzerland) equipped with a Rockwell diamond tip of 200 μm radius. Scratch resistance is measured as the instantaneous penetration depth R_p and 2 min later as the residual or healing depth R_h along grooves 5.0 mm long at a scratching speed of 5.33 mm/min. Results were obtained for scratching under applied loads between 0.03 and 10 N.

Scanning electron microscopy

The GCC scaffold morphology was analysed by freeze fracture with a JEOL JSM-5900LV scanning electron microscopy (SEM). The instrument operated at 15 kV of accelerating voltage and 21 mm work distance.

### Membrane contact angle measurements

The contact angles of the thermal remoulding membranes were studied by goniometry using the static water drop method. The water drop (7 μL) was carefully placed by a pipette on the membrane surface, photographed and later evaluated by the ImageJ program to determine the contact angles.

Membranes' morphology

The morphology of the surfaces of the thermally remoulded membranes was examined in tapping mode with a JEOL JSPM 5200 Atomic Force Microscopy (AFM) coupled to Energy Dispersive X-ray Spectroscopy (EDS). The machine was equipped with a silicon nitride cantilever (Micromash). The samples were studied without coating.

Results and discussion

An outline of the reaction of grafting of CS onto P(3HB) is shown in Figure 1. The mechanism of the reaction was studied before.[15] However, the ^{1}H/ solid-state ^{13}C CP-MAS NMR and FTIR spectroscopies were performed only later to evaluate the success, or otherwise, of the grafting. The proposed
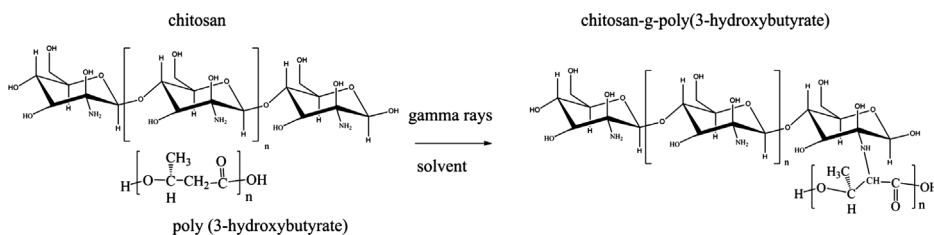
1 Structures of chitosan, poly(3-hydroxybutyrate) and proposed structure of P(3HB-g-CS)

structure is also consistent with the investigations on graft polymerization of styrene monomers onto chitosan initiated by gamma ray irradiation,[18] and the grafting of vinyl butyrate onto chitosan by a chemical initiator (potassium persulfate).[19]

Figure 2 shows the solid state spectra of ^{13}C-NMR (a) and the ^{1}H-NMR (b) of chitosan-g-poly (3-hydroxybutyrate) and P(3HB), respectively. We see in Figure 2a that the sharp signals belong to P(3HB), while the broad ones from 50 to 120 ppm are correlated to the CS moieties. The results obtained are consistent with the synthesis of PHA conjugates by amidation reported by Yalpani et al.[20]

Figure 2b, ^{1}H-NMR of P(3HB-g-CS), shows new broad signals from the region 3.7–4.8 ppm for the grafted P(3HB). Peaks that appear at 3.90 and 4.21 ppm belong to the CH new protons, a consequence of grafting. These signals are broad as indicative of polymerization, in agreement with the results obtained by Sarkar et al.[21] in grafting of low molecular weight N-maleated chitosan onto polyamidoamine and also with grafting of n-dextran onto CS.[22] We find that CS has been successfully grafted onto P(3HB).

IR spectra of P(3HB-g-CS), P(3HB) and CS are shown in Figure 3. The grafted polymer has the P(3HB) and CS characteristic signals, but some new features are seen: (a) compared with CS spectrum, the P(3HB-g-CS) shows a broad band centered at 3367.5 cm^{-1}, attributed to the N–H stretching and less intense than the one for CS; (b) the OH stretching overlaps with N–H stretching; (c) signals at 2981.5, 2931.4 and 2872.4 cm^{-1}, assigned to stretching of carbons SP$_3$–H and SP$_2$–H; (d) the carbonyl stretching at 1722.7 cm^{-1} is seen as a strong signal in the graft copolymer, (d) around 1577 cm^{-1} signals representing the N–H bending vibration of primary amine. This indicates that some CS remains ungrafted.

A more unusual feature for chitosan are two bands in the 3360 cm^{-1} region, while the P(3HB-g-CS) spectrum has only one band. This is characteristic of secondary amines and strongly supports the grafting by the NH$_2$ groups, although the overlapping with the alcohol group makes difficult to reject the possibility of grafting by the hydroxyl group. Thus the radiation-induced graft polymerization of chitosan onto P(3HB) is proven by this technique. The results obtained are in accordance with those for from the graft polymerization of 4-(6-methacryloxyhexyloxy)-4′-nitrobiphenyl,[23] poly(ethylene glycol),[24] and acrylamide onto chitosan.[25]

Thermal properties of materials obtained from different solvents are shown in Figure 4. The derivative curves for P(3HB-g-CS) show that thermal stability of the grafted polymers is lower than that of chitosan (Figure 4a) but higher than that of P(3HB) (Figure 4b). After grafting, the products exhibit two regions of decomposition temperatures (Figure 4c), the first region at 240–280 °C and the second at 285–310 °C. The first thermal decomposition step is probably due to the P(3HB) decomposition (Figure 4b), and the second may be due to the degradation of CS. The degree of grafting DG/(%) values obtained from Equation (1) are listed in Table 1. The DG/(%) range is between 14 and 32%. The highest value is obtained for P1Q1S3 (32.1%) and the lowest one for P1Q1S6 (14.3%). An increase in the degree of grafting lowers the decomposition temperature (shown as T_d in Table 1) and the onset temperature (T_{onset}). Our results are also congruent with those for the grafting of 1-cyanoethanoyl-4-acryloylthiosemicarbazide,[26] and poly (2-(furan-2-carbonyl)-acrylonitrile) onto CS.[27]

Figure 5 shows the scratch resistance determination results for the pristine and grafted P(3HB). We evaluate the penetration and recovery depth of the indenter during and after the scratch. The sample is mounted on a table which measures the surface topography during a scratch test.[28, 29]
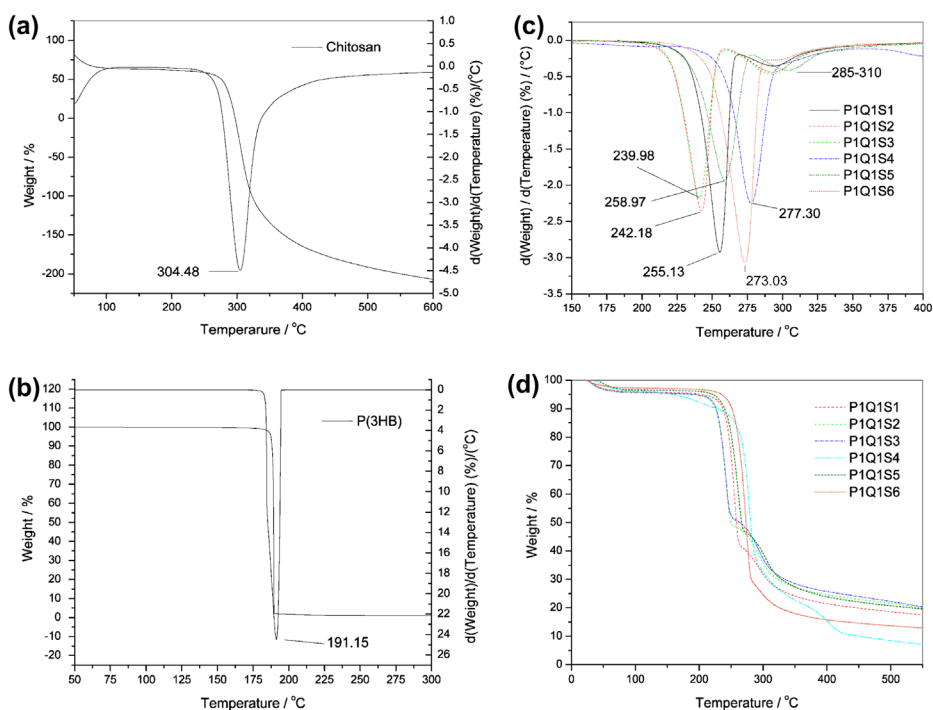
The penetration depth, R_p, is measured during the movement of the diamond indenter. The residual depth (recovery depth), R_h, is measured in the post-scan mode. The percentage of viscoelastic recovery f is calculated[29] as
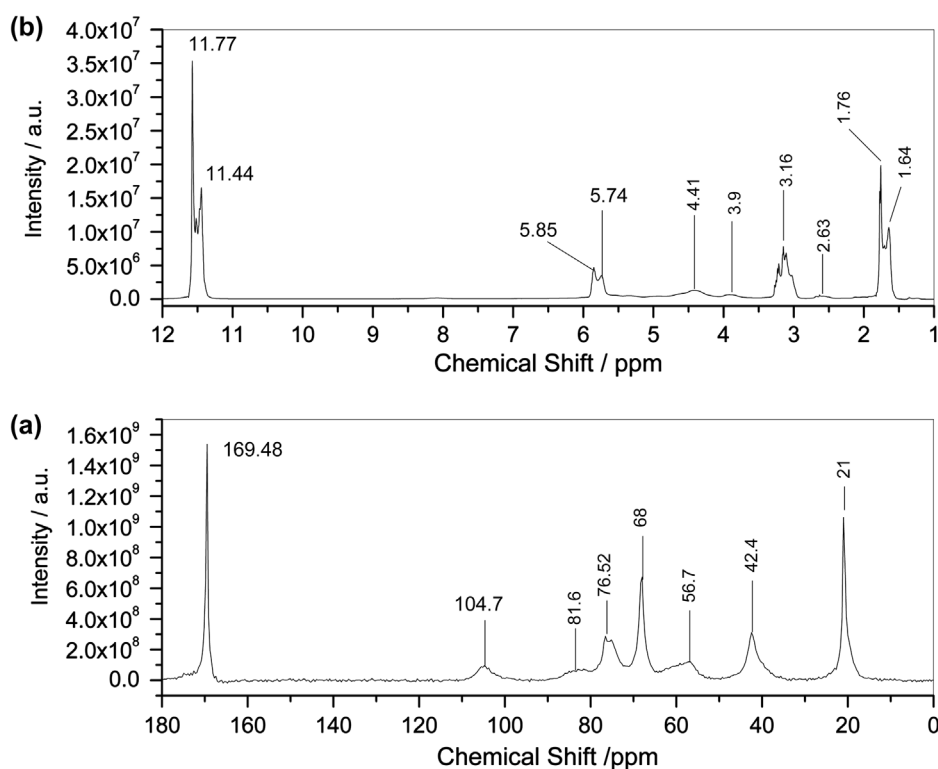
2 Bottom: solid-state 13C-NMR of P(3HB-g-CS) *a*; Top: 1H-NMR of P(3HB-g-CS) *b*
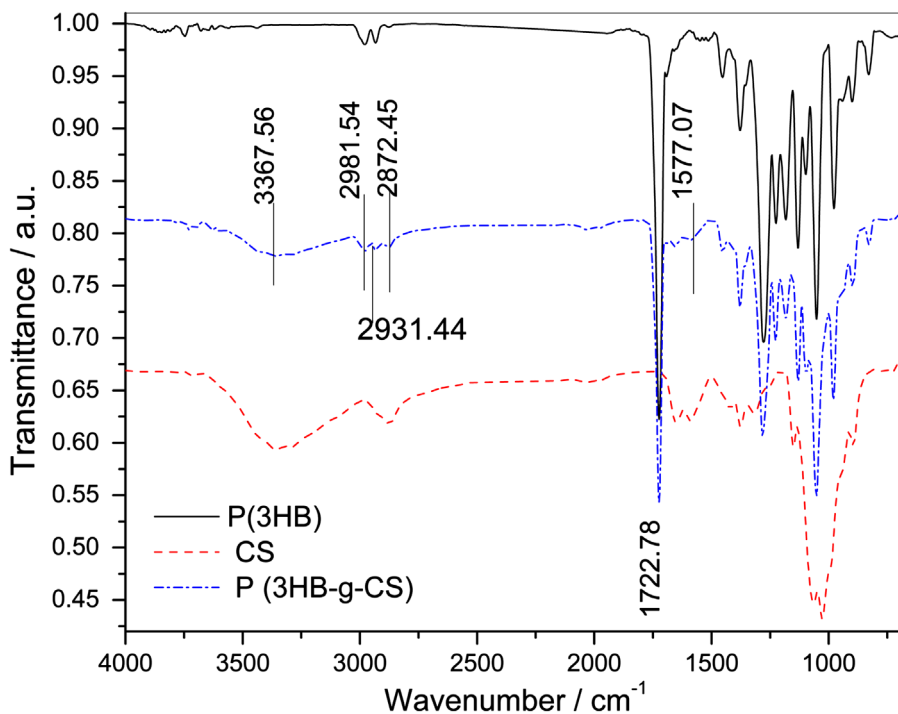
**3 FTIR spectroscopy of P(3HB), chitosan and chitosan-g-poly(3-hydroxybutyrate)**

4 Thermograms (TGA and DTGA): *a* top left: chitosan; *b* bottom left: poly(3-hydroxybutyrate); *c* top right: derivative thermogravimetric curves of P(3HB-g-CS) obtained in different solvents; *d* bottom right: TGA of grafted polymers (P(3HB-g-CS))

Table 1 Thermodynamic parameters from the P(3HB-g-CS) calorimetry

Sample	DG (%) (TGA)	Td (°C)	T_{onset} (°C)
P0	0	191.2 ± 0.2[a]	186.7 ± 0.2[a]
Q0	0	304.5 ± 0.2[a]	287.7 ± 0.2[a]
P1Q1S1	22.8 ± 0.2[a]	255.4 ± 0.2[a]/292.8 ± 0.2[a]	242.5 ± 0.2[a]
P1Q1S2	29.3 ± 0.2[a]	242.2 ± 0.2[a]/292.7 ± 0.2[a]	229.6 ± 0.2[a]
P1Q1S3	32.1 ± 0.2[a]	239.9 ± 0.2[a]/292.7 ± 0.2[a]	227.2 ± 0.2[a]
P1Q1S4	15.9 ± 0.2[a]	277.3 ± 0.2[a]/402.4 ± 0.2[a]	269.5 ± 0.2[a]
P1Q1S5	27.2 ± 0.2[a]	259 ± 0.2[a]/303 ± 0.2[a]	234 ± 0.2[a]
P1Q1S6	14.3 ± 0.2[a]	273 ± 0.2[a]/297 ± 0.2[a]	248.8 ± 0.2[a]

[a]The results are the mean ± standard deviation for three separate experiments.

(a)

(b)

(c)
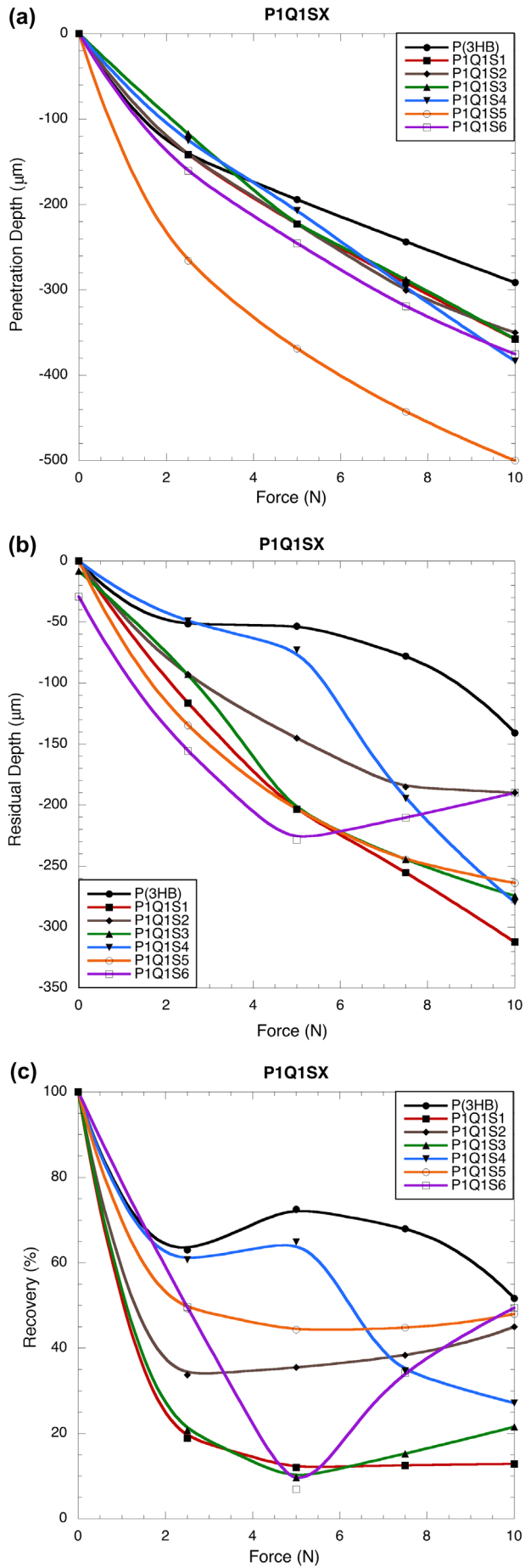
5 *a* Single scratch penetration depths; *b* residual depth, and *c* recovery of neat P(3HB) and P(3HB-g-CS) as function of the applied force
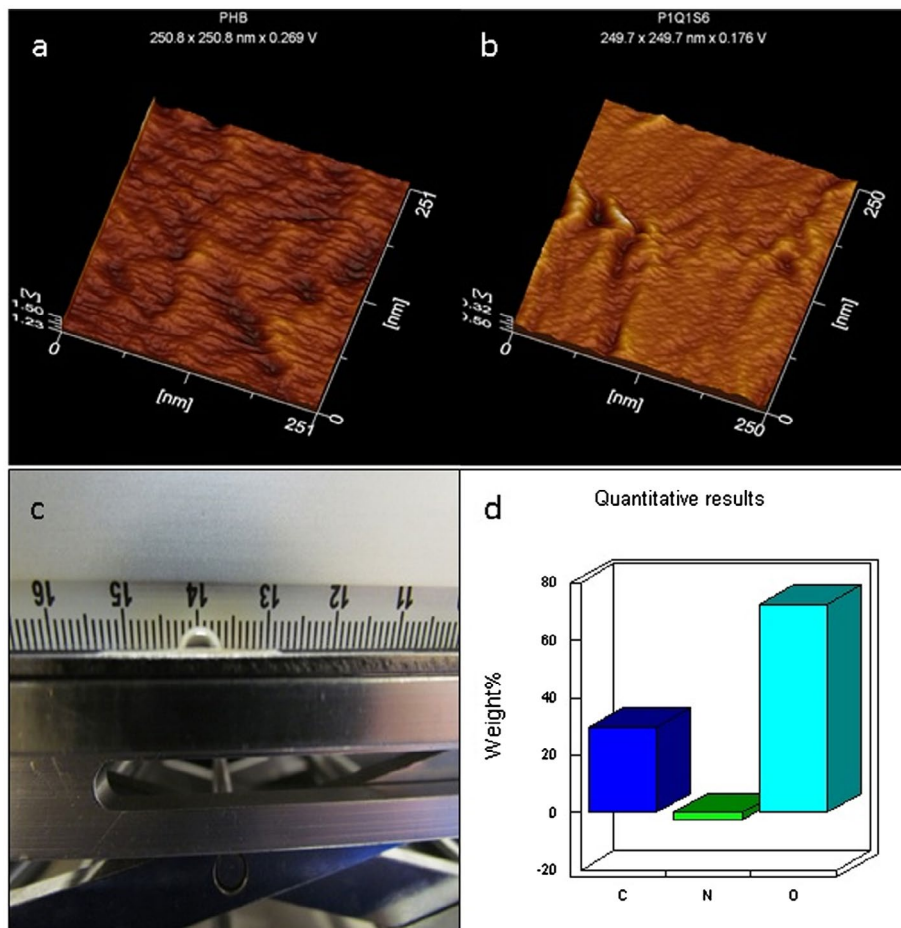
6 *a* top left: AFM of P(3HB) membrane surface; *b* top right: AFM of P(3HB-g-CS) membrane surface; *c* bottom left: drop photography from the contact angle study; *d* bottom right: image of the EDS concentration analysis of the content of P(3HB-g-CS) membrane surface
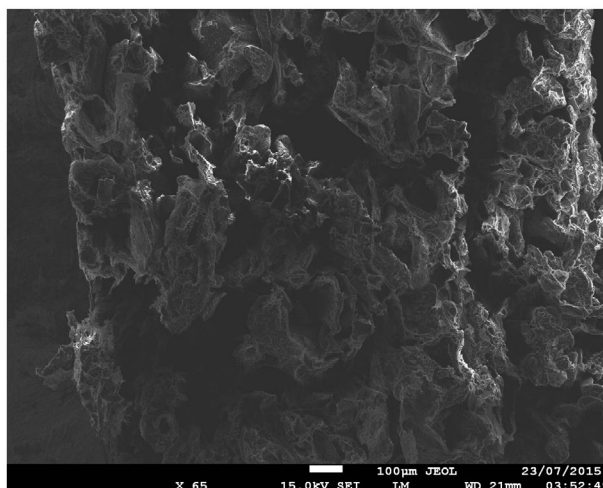
7 SEM micrograph of porous P(3HB-g-CS) GCC scaffolds

$$f = [(R_p - R_h)100\%]/R_p \qquad (2)$$

We have chosen 0.03–10.0 N as the loading range because of the thickness (<0.5 mm) of samples. P(3HB) has the highest resistance to scratching. The P(3HB-g-CS) samples were brittle and easy to tear or crack during the testing. Hence not fully reliable results are not included. P(3HB) exhibits the highest recovery.[30–32]

We conclude that membranes obtained by thermal remoulding would be unsuitable for molecular linkage of proteins or other substrates. The grafting occurs mostly in the amino groups, reducing their availability to react with

other compounds. Thermal analysis shows that the materials obtained have a lower thermal stability than the source materials. When preparing the membranes, the grafting copolymer was partially degraded; this explains why the surfaces of the membranes, studied by AFM (Figure 6*a* and *b*) and by contact angles (Figures 6*c*), show only insignificant variations with the degree of grafting and between neat and grafted copolymer. The membranes were also studied by EDS mapping to verify that the amino groups could not be easily detected on surfaces (Figure 6*d*). Membranes were also prepared by solvent casting and were found to be easily breakable.

We have also examined the GCC scaffolds by SEM. Figure 7 shows the SEM of the freeze-dried broken GCC scaffolds. The size of the pores ranges from 100 to 250 μm, with porosity and mean pore size of 80% and 125 μm, respectively. Such parameters are indicative of good scaffolds for cell/tissue ingrowth.[33] However, further work is needed to evaluate the relationships of the porosity and pore size of our samples with the composite concentration of pore former and the polyurethane.[34,35] Our GCC scaffolds can potentially be used in tissue engineering.

A survey of results

We have demonstrated that chitosan can be successfully grafted onto P(3HB) by radiation-induced polymerization. Both NMR and FTIR confirm the successful grafting, apparently by the NH_2 groups of the chitosan molecule. P(3HB-g-CS) is less thermally stable than the pristine samples. New membranes show less resistance to scratching. The AFM images reveal that the surface is like that of P(3HB), probably due to degradation during the membrane synthesis process. The synthesized grafting membranes are unsuitable for intended applications. On the other hand, the GCC scaffolds have satisfactory morphological characteristics such as size, degree of porosity and mean pore size. Hence, they can be useful for cell/tissue ingrowth.

Acknowledgements

ORCID

M. González http://orcid.org/0000-0003-3899-4410

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