Synthesis, characterization and antimicrobial activity of biguanidinylated chitosan-g-poly[(R)-3-hydroxybutyrate]

Hend E. Salama, Gamal R. Saad, Magdy W. Sabaa*

Department of Chemistry, Faculty of Science, Cairo University, 12613, Egypt

A R T I C L E   I N F O
Article history:
Received 14 October 2016
Received in revised form 14 December 2016
Accepted 14 March 2017
Available online 18 March 2017

Keywords:
Chitosan biguanidine
Poly[(R)-3-hydroxybutyrate]
Antimicrobial activity

A B S T R A C T
Chitosan biguanidine hydrochloride (ChG) and low molecular weight poly[(R)-3-hydroxybutyrate] (PHB) were successfully prepared to overcome the solubility problem of chitosan and PHB and also to enhance antimicrobial activity of chitosan. The graft copolymers based on ChG and PHB (ChG-grafted PHB) were then prepared via condensation reaction of the carboxylic groups of PHB with the amino groups of ChG. These graft copolymers swell in water. The prepared graft copolymers were characterized by FTIR, 1H NMR, X-ray diffraction (XRD) and thermal analyses (TGA and DSC). TGA and DSC results revealed that the thermal stability and crystallinity of the graft copolymers were found to increase as the content of PHB increased. The antimicrobial activity of both ChG and ChG-grafted PHB, against Strep tokococcus pneumoniae, Bacillus subtilis, Escherichia coli (as examples of bacteria) and Aspergillus fumigatus, Geotrichum candidum and Syn cephalastrum recte nomus (as examples of fungi), were tested. Among them, ChG and ChG-grafted PHB with the highest grafting percent investigated showed to possess relatively higher antimicrobial activity with low MIC values in the range of 0.49–3.90 μg mL⁻¹.

© 2017 Elsevier B.V. All rights reserved.

1. Introduction

Chitosan is a linear polysaccharide, composed of glucosamine (GlcN) and N-acetyl glucosamine units linked by β-(1,4) glycosidic bonds. It is mainly obtained by deacetylation of chitin under alkaline conditions [1,2]. It is a semi-crystalline weak base polymer, insoluble in water, alkali or aqueous solutions above pH 7, and common organic solvents due to its stable and rigid crystalline structure. Beside its biodegradability and biocompatibility, chitosan is reported to be an active polymer with antimicrobial activity [3–5]. The cationic nature of chitosan makes it a polymer of high importance from environmental and biomedical points of view, among other natural polysaccharides. However, chitosan shows its antimicrobial activity only in acidic medium, because of its poor solubility above pH 6.5. In principle, chitosan can kill some kinds of microbes by disrupting their normal structures due to the reaction between the positive charges of chitosan and the negatively charged cell walls of bacteria or its inner proteins [6]. To overcome some of the disadvantages of the pristine chitosan, it is most imperative to functionalize it with suitable functional groups. In principal, graft copolymerization may be considered as a good approach to achieve novel types of tailored hybrid chitosan-based materials with improved properties and thus widening their applications in biomedicine and environmental fields. Grafting of chitosan allows the formation of functional derivatives by covalent binding of a grafted molecule into the chitosan backbone. In the literature, several studies were reported about grafting on the chitosan's chains; such as grafting of poly(4-vinylpyridine) [7,8], polyacrylamide [9,10], poly(acrylic acid) [11], poly(vinyl alcohol) [12], poly(ethylene glycol) [13–17], poly(lactic acid) [18,19], poly(e-caprolactone) [20,21] and poly(3-hydroxyalkanoate) [22,23].

Guaindine is an important nitrogen-containing functionality and characteristic structural motif which commonly exists in biologically active compounds and in a great number of natural compounds that carry out different and important functions in animals and plants [24,25]. Since guanidine group seems to possess the strongest positive charge (pKa = 12.5) in acidic, neutral or basic solutions; therefore guanidine derivatives are considered as antibacterial and antiviral materials. Recently, a number of polymers bearing guanidine groups, exhibiting excellent antimicrobial properties and high water solubility, have been investigated [26–30], including chitosan [31–42]. Attaching the guanidine group onto chitosan introduces positive charge onto the polymer back-
bone, results in a better aqueous solubility at neutral pH and shows potentially good antimicrobial activity. Such modified chitosan derivatives would contain a highly polar group that is also multifunctional.

On the other hand, poly(3-hydroxybutyrate) (PHB) is a biodegradable polyester produced by a number of bacteria as a reserve of carbon and energy. It has many unique properties, such as good biodegradability, nontoxicity, and biocompatibility with tissue and blood [43,44]. Therefore, it has been used for biomedical applications including tissue engineering and bone replacement materials, and also used as packaging materials and personal disposable articles [43,45].

Unfortunately, PHB suffers from some disadvantages in comparison with conventional thermoplastics, such as brittleness, narrow processing window, and of relatively high price. PHB is a highly crystalline polymer; the growth of crystals within the large spherulites is the origin of its brittleness [46]. The thermal instability of PHB is ascribed to its easy decomposition at a temperature slightly above its Tm by a β-hydrogen elimination mechanism. These shortcomings seriously limit its widespread commercial use.

Copolymer based on chitosan and PHB is expected to be environmentally friendly materials which have complete biodegradability and possess other properties suitable for biomedical applications [47,48]. This study aims to graft chitosan with low molecular weight poly[(R)-3-hydroxybutyrate] to combine their advantages. First, chitosan biguanidine is prepared, aiming to enhance the antimicrobial activity of chitosan and overcome its limited solubility in organic solvents, then grafted with end carboxyl group of PHB prepolymers. The prepared copolymers are investigated for their molecular structures, physical properties and antimicrobial activities.

2. Materials and methods

2.1. Materials

Chitosan from crab shell was purchased from Oxford laboratory (India). Its degree of deacetylation (DD) is 94%, as determined by potentiometric titration [49] and the viscosity-average molecular weight is 160,000 g mol⁻¹. Bacterial poly[(R)-3-hydroxybutyrate], PHB, was obtained from Coper sucar, Piracicaba, Brazil. All other chemicals were of reagent grade and used as received.

Streptococcus pneumoniae (S. pneumoniae, RCMB 010010) and Bacillus subtilis (B. subtilis, RCMB 010067) as Gram-positive bacteria, Escherichia coli (E. coli, RCMB 010052) as Gram-negative bacterium, Aspergillus fumigatus (A. fumigatus, RCMB 02568), Geotricum candidum (G. candidum, RCMB 05097) and Syncephalastrum racemosum (S. racemosum, RCMB 05922) as fungi, were provided by the regional center for mycology and biotechnology, El-Azhar University, Egypt.

2.2. Synthesis of chitosan biguanidine hydrochloride (ChG)

Chitosan (1.0 g) was dissolved in 100 mL of 1 wt.% hydrochloric acid at room temperature under continuous stirring for 3 h; then 0.52 g of cyanoguanidine (corresponding to a molar ratio of 1:1 compared with repeating unit of chitosan) in 20 mL H₂O was added dropwise to chitosan solution. The reaction mixture was then stirred at 100 °C for 3 h, then cooled to room temperature and precipitated in excess methanol, filtered and dialyzed against distilled water for one day to remove the un-reacted reagent. The obtained product was dried in vacuum at 80 °C for 24 h [32,34]. The route of the reaction is shown in Scheme 1.

2.3. Preparation of PHB prepolymer

Low molecular weight PHB was prepared according to the method described before [50]. PHB (0.5 g) was placed in 100 mL round-bottomed flask with 50 mL of 3 N HCl and refluxed at a temperature of 104.5 °C for 24 h. The hydrolyzed product was then rapidly cooled to room temperature by immersing the flask in ice-water bath. The acid suspension was then repeatedly washed and centrifuged (50,000 rpm) three times in distilled water and twice in ethanol. The final product was then dried at 60 °C overnight. The weight average molecular weight of the obtained PHB prepolymer was determined by gel permeation chromatography (GPC) and found to be 13,000 g mol⁻¹.

2.4. Preparation of ChG-grafted PHB copolymers

To 1.3 g of ChG dissolved in 35 mL DMSO, different amounts of PHB (0.25–1.0 g) dissolved in 30 mL of CH₂Cl₂/DMSO (1/2, v/v) was added drop wise with continuous stirring for 4 h at room temperature. The reaction mixture was stirred at 35 °C for further 24 h; then precipitated in distilled water, filtered and dried in vacuum oven at 60 °C. The obtained product was then placed in chloroform for 24 h to remove the un-reacted PHB. The final product was washed with acetone, filtered and dried at 60 °C for 48 h [47,51]. The route of the synthesis is given in Scheme 1. Three samples of ChG-grafted PHB copolymers with different content of PHB were prepared, coded here as, ChG-PHB1, ChG-PHB2 and ChG-PHB3.

3. Characterization and analysis

3.1. Infrared spectroscopy

FTIR spectra of the chitosan and chitosan derivatives were recorded in the frequency range of 400–4000 cm⁻¹ using a Perkin Elmer B25 spectrophotometer. Powder samples were mixed with KBr (50–200 mg), and the mixtures were pressed into disks for measurements. All measurements were carried out with 64 scans at resolution of 2 cm⁻¹ at room temperature.

3.2. ¹H NMR

¹H NMR spectra were recorded on a Bruker AC-400 in DMSO-d₆, DMSO-d₆/CF₃COOD and CDCl₃ as solvents.

3.3. X-Ray diffraction

X–Ray diffractograms of the investigated samples were obtained using an X–ray powder diffractometer (a Philips Xpert MPD Pro) with Ni – filter and Cu Kα radiation source at an accelerating voltage/current of 50 kV/40 mA. The relative intensity was recorded in the scattering range 2θ, varying from 3° to 50° at scanning rate 2° min⁻¹.

3.4. TGA analyses

TGA curves of the investigated samples were conducted using Shimadzu TGA 50H Thermal Analyzer under nitrogen atmosphere with a dynamic heating rate of 10 °C min⁻¹. All experiments were conducted from room temperature to 800 °C and the reference material was alumina. Samples weights, in all experiments, were taken in the range of 4–5 mg.

3.5. Differential scanning calorimetry

Differential scanning calorimetry (DSC) was carried out using a TA Instruments Co. Q200 Differential Scanning Calorimeter (DSC;
USA). The DSC was calibrated using the melting temperature and enthalpy of indium and lead. DSC investigation was carried out for powder samples (8–10 mg) under a purge of nitrogen. For each sample, the following thermal cycle was applied: a first scan was made from 20 to 180 °C at a heating rate of 10 °C min⁻¹, then kept for 3 min at 180 °C, and then cooled to 30 °C at a cooling rate of 10 °C min⁻¹; and finally reheated again up to 180 °C.
3.6. Grafting percentage and solubility test

The grafting reactions were carried out with various PHB feed amounts, as shown in Table 1. The content of PHB in the grafted copolymers was calculated using the following Equation [22]:

\[
\text{PHB grafting percentage} = \frac{\text{Initial weight of PHB (g)} - \text{Unreacted PHB (g)}}{\text{Yield (g)}} \times 100
\]

The determined compositions of the copolymers are included in Table 1.

The solubility of chitosan, ChG and ChG-grafted PHB copolymers in different solvents are presented in Table 1. It can be seen that the solubility of the graft copolymers differ from that of pure chitosan or ChG. The chitosan and ChG are soluble in an acidic aqueous solution, whereas the prepared CHG-grafted PHB copolymers only swell. The graft copolymers, irrespective of its PHB content, swell in distilled water. In addition, ChG as well as CHG-grafted PHB copolymer exhibit solubility in DMSO. This difference in solubility confirms that a chemical modification is occurred on the chitosan’s chains.

3.7. Antimicrobial measurements

Antimicrobial activity of the Ch, ChG and ChG-grafted PHB copolymers was determined using agar well diffusion method [52]. All the materials were tested in vitro for their antibacterial activity against Streptococcus pneumoniae (S. pneumoniae, RCMB 010010) and Bacillus subtilis (B. subtilis, RCMB 010067) as Gram-positive bacteria, and against Escherichia coli (E. coli, RCMB 010052) as Gram-negative bacteria using nutrient agar medium. Antifungal activity was carried out against Aspergillus fumigatus (A. fumiga- tus, RCMB 02568), Geotrichum candidum (G. candidum, RCMB 05097) and Syncphalastrum recemosum (S. recemosum, RCMB 05922) using Sabouraud Dextrose Agar medium. Ampicillin, gentamicin and amphotericin B were used as standard drugs for Gram-positive, Gram-negative and antifungal activity, respectively. In brief, 5 mL of the sterilized medium (prepared by dissolving 10 g tryptone, 5 g yeast extract and 10 g sodium chloride in 1000 mL deionized water) were poured onto the sterilized Petri dishes (20–25 mL, each Petri dish) and allowed to solidify. Wells of 6 mm diameter was made in the solidified media with the help of sterile borer. A sterile swab was used to evenly distribute microbial suspension over the surface of solidified media and 0.1 mL of different concentrations of the tested materials was added to each well with the help of micropipette. The plates were incubated at 37 °C for 24 h in case of antibacterial activity and at 25 °C for 48 h in case of antifungal activity. A blank without the tested materials was prepared for comparison. Zones of inhibition were estimated by measuring the diameter of the bacterial or fungal growth inhibition zone. The values were taken as the average of three independent experiments.

The minimum inhibitory concentration (MIC) was determined by the brain heart infusion broth micro dilution method using 96-well micro-plates [53,54]. The inoculated of the microbial strains was prepared from 24 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity (10^5 CFU mL⁻¹). Each sample (1.0 mg) was suspended in DMSO (1 mL) to obtain 1000 μg mL⁻¹ stock solution. Positive and negative controls were included in each plate. Sterile broth (100 μL) was added to the well from row B to H. The stock solutions of samples (100 μL) were added to the wells in rows A and B. Then, the mixture of samples and sterile broth (100 μL) in row B were transferred to each well in order to obtain a twofold serial dilution of the stock samples (concentrations; 500, 250, 125, 62.5, 31.30, 15.60, 7.81, 3.90, 1.95, 0.98 and 0.49 μg mL⁻¹). The inoculums (100 μL) were added to each well and a final volume of 200 μL was obtained in each well. Plates were incubated at 37 °C for 24 h in case of antibacterial activity and at 25 °C for 48 h in case of antifungal activity. Microbial growth was indicated by the presence of turbidity and a pellet at the bottom of the well. The optical density of each well was measured using ELISA reader device set at 520 nm.

3.8. Statistical analysis

The data were analyzed using statistical software packages of SPSS® software (Version 16, SPSS Inc., Chicago, IL) with a one-way analysis of variance (ANOVA) and the independent student T-test were done.

4. Results and discussion

4.1. Characterization of ChG-grafted PHB copolymer

4.1.1. Chemical structure

Chemical structure of the prepared ChG-grafted PHB copolymers was elucidated by FTIR and 1H NMR spectra. Fig. 1 shows FTIR spectra of chitosan (Ch), ChG, PHB and ChG-PHB2 graft copolymer. In the FTIR spectrum of Ch: the main characteristic bands could be assigned as follows: 3444 cm⁻¹ (−OH and −NH₂ stretching vibration) [55], 2948–2866 cm⁻¹ (−CH stretching vibration in –CH and –CH₂), 1075 cm⁻¹ (−O stretching vibration), 1660 and 1597 cm⁻¹ (amide I & II, respectively). For CHG, new bands at 1630 (C≡N− stretching vibration), 1532 cm⁻¹ (C=NH₂ stretching vibration) and 1468 cm⁻¹ (C–N stretching vibration of the guanidinium group) appeared. These results revealed that the guanidinylation of chitosan occurs successfully. By comparing the spectra of CHG and PHB with that of CHG-PHB2, it was found that the band at 1585 cm⁻¹ (due to −NH₂ bending vibration) of CHG disappears, while the intensity of the band at 1626 cm⁻¹ (which is due to C−N stretching vibration in addition to amide bond stretching vibration which results from the reaction between carboxylic group of PHB and amino groups of CHG) is increased. Furthermore, the band at 1729 cm⁻¹, which is assigned to stretching vibration of the ester carbonyl group of PHB repeating units, appeared indicating that PHB was successfully grafted onto chitosan biguanidine.

Fig. 2 shows 1H NMR spectra of Ch, ChG, PHB, and CHG-PHB2. All the spectra exhibited the characteristic 1H NMR pattern of chitosan, i.e., the multiplet at δ 3.3–3.9 ppm which is due to H5, H4, H3 and H6, and the singlet at δ 2.8 ppm, due to C2 protons of the N-glucosamine and N-acyetyl-glucosamine, and at 1.9 ppm due to the N-acyetyl protons of N-acyetyl-glucosamine [56]. The multiplet at δ 4–5.5 ppm is due to H1 and OH (at C3 and C6), the broad singlet at 8.2 ppm is due to (NH3/NH) [57]. As seen in Fig. 2, the CHG has new signals at 7.1 ppm, 8.5 ppm and 10.4 ppm which are assigned to the protons of the biguanidinium group [58]. Appearance of these characteristic signals supported the formation of biguanidylated chitosan [42].

By comparing the signals of the CHG-PHB2 graft copolymer with that of ChG and PHB, the signals at 5.2 and 1.2 ppm, assigned to the methine (−OCH2CH2CH2CO)− and methyl (−OCH2CH2CH2CO−) protons of the PHB repeating units, respectively, were observed confirming that the PHB had been grafted successfully onto the CHG base polymer.

The content of the PHB in the CHG-grafted PHB copolymers were also determined using 1H NMR from the peak intensities at 2.8 ppm for the H2 proton of the chitosan unit and between 5.0–5.3 ppm for the methine proton of PHB unit together with other pyranose protons using the following equation [15].

\[
\text{PHB grafting percentage} = \frac{86}{161} \times \left( \frac{15.0–5.3 - 51.28}{42.8} \right) \times 100
\]

where 86 and 161 are the molecular weights of the units of PHB and the chitosan, respectively.
Table 1

<table>
<thead>
<tr>
<th>Samples code</th>
<th>Initial condition</th>
<th>Yield (g)</th>
<th>Soluble fraction of PHB in CHCl$_3$ (unreacted PHB) (g)</th>
<th>Grafting%</th>
<th>Solubility characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChG-PHB1</td>
<td>1.30</td>
<td>0.25</td>
<td>1.46</td>
<td>0.02</td>
<td>PHB wt.% in copolymer</td>
</tr>
<tr>
<td>ChG-PHB2</td>
<td>1.30</td>
<td>0.50</td>
<td>1.65</td>
<td>0.05</td>
<td>DMSO</td>
</tr>
<tr>
<td>ChG-PHB3</td>
<td>1.30</td>
<td>1.00</td>
<td>1.95</td>
<td>0.25</td>
<td>Water</td>
</tr>
<tr>
<td>Ch</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2% AcOH</td>
</tr>
<tr>
<td>ChG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ethanol</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Acetone</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ChG</td>
<td>15.7</td>
<td>17.5</td>
<td>+</td>
<td>±±</td>
<td>±</td>
</tr>
<tr>
<td>ChG-PHB1</td>
<td>27.3</td>
<td>22.3</td>
<td>+</td>
<td>±±</td>
<td>±</td>
</tr>
<tr>
<td>ChG-PHB2</td>
<td>35.5</td>
<td>33.5</td>
<td>+</td>
<td>±±</td>
<td>±</td>
</tr>
<tr>
<td>ChG-PHB3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>±</td>
</tr>
<tr>
<td>Ch</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>ChG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>ChG-PHB1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>−</td>
</tr>
<tr>
<td>ChG-PHB2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>−</td>
</tr>
<tr>
<td>ChG-PHB3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>−</td>
</tr>
<tr>
<td>Ch</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>−</td>
</tr>
<tr>
<td>ChG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>−</td>
</tr>
</tbody>
</table>

a) PHB grafting percentage calculated theoretically from initial weight of used PHB, weight of unreacted PHB and yield as (Initial weight of PHB (g) – Unreacted PHB (g))/Yield (g) × 100).

b) PHB grafting percentage calculated from $^1$H NMR.

Solubility in different solvents (+, soluble; ±±, highly swelled; ±, swelled; and −, insoluble).

4.1.2. X-ray diffraction pattern

Fig. 3 represents the X-ray diffraction patterns of Ch, ChG, PHB and ChG-grafted PHB copolymers. In XRD pattern of chitosan, the diffraction maxima around 10.1° and 20.4° are attributed to the (020) and (110) planes of the crystalline lattice with inter-plane distances of 8.8 and 4.6 Å, correspondingly. These results suggested the formation of inter- and intra-molecular hydrogen bonds in presence of the free amino groups in chitosan. For ChG, the diffraction peaks around 10.1° and 20.4° are almost disappeared, and new diffraction peaks around 27° and 31° were observed. As a result, the ChG sample exhibited lower crystallinity compared with Ch. This could be ascribed to the presence of biguanidinium side chains which will certainly hinder the formation of inter- and intra-molecular hydrogen bonds. X-ray diffraction peaks of PHB appeared at 2θ values of 13.4°, 17°, 22.5° and 25.5° are assigned to (0 2 0), (1 0 0), (2 0 0) and (1 0 1) of the orthorhombic unit cell, respectively [59,60]. ChG-grafted PHB samples, showed characteristic peaks of PHB component compared with neat PHB, suggesting that chitosan and PHB grafted chain were mixed at a molecular level and the ChG-grafted PHB copolymers exhibited somewhat a crystalline fraction.

4.2. Thermal studies

4.2.1. Thermal stability

TGA curves of Ch, ChG and ChG- grafted PHB copolymers are shown in Fig. 4. The thermal characteristics; namely, the initial decomposition temperature ($T_d$) and the temperature at which maximum rate of decomposition occurred for the second and third steps $T_{d1}$ and $T_{d2}$ are summarized in Table 2. All the tested samples gave three stages of mass loss. The first stage occurred in the temperature range 25–170 °C (ca. 7–14% mass loss) is ascribed to the evaporation of the adsorbed water. For chitosan, the second mass loss started at 253 °C and continued up to 369 °C with a maximum rate at 296 °C, during which there was 45% mass loss is due
to further dehydration, deacetylation and degradation of chitosan [61–63], while the third one within 369–599 °C (ca. 45% mass loss) with a maximum rate at 535 °C corresponds to the residual cross-linked degradation of chitosan [64]. The TGA of the ChG and neat chitosan exhibited similar profiles, but the second mass loss of ChG was observed to occur in the temperature range 155–330 °C with
Table 2
Thermal properties of ChG, PHB and ChG-grafted PHB copolymers.

<table>
<thead>
<tr>
<th>Samples code</th>
<th>( T_i (\degree C) )</th>
<th>( T_{max1} (\degree C) )</th>
<th>( T_{max2} (\degree C) )</th>
<th>( T_{evap} (\degree C)^{a} )</th>
<th>( \Delta H_{evap} (\mathrm{J} \cdot \mathrm{g}^{-1})^{b} )</th>
<th>( T_m (\degree C)^{b} )</th>
<th>( \Delta H_m (\mathrm{J} \cdot \mathrm{g}^{-1})^{b} )</th>
<th>( T_{rec} (\degree C) )</th>
<th>( \Delta H_{rec} (\mathrm{J} \cdot \mathrm{g}^{-1}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>( - )</td>
<td>( - )</td>
<td>( - )</td>
<td>( - )</td>
<td>( - )</td>
</tr>
<tr>
<td>ChG</td>
<td>177</td>
<td>225</td>
<td>668</td>
<td>79</td>
<td>209</td>
<td>167</td>
<td>87</td>
<td>116</td>
<td>84</td>
</tr>
<tr>
<td>ChG-PHB1</td>
<td>199</td>
<td>235</td>
<td>277</td>
<td>62</td>
<td>128</td>
<td>163</td>
<td>34</td>
<td>103</td>
<td>30</td>
</tr>
<tr>
<td>ChG-PHB2</td>
<td>200</td>
<td>224</td>
<td>281</td>
<td>51</td>
<td>58</td>
<td>164</td>
<td>45</td>
<td>113</td>
<td>42</td>
</tr>
<tr>
<td>ChG-PHB3</td>
<td>205</td>
<td>212</td>
<td>281</td>
<td>51</td>
<td>51</td>
<td>167</td>
<td>51</td>
<td>116</td>
<td>48</td>
</tr>
</tbody>
</table>

\(^{a}\) Not present.
\(^{b}\) Determined from the DSC first heating scan.

Fig. 3. XRD patterns of Ch, ChG, PHB and ChG-grafted PHB copolymers.

a maximum rate at 225 \(^\degree\)C (ca. 48% mass loss). This decomposition step corresponds to the dehydration and degradation of the chitosan base polymer together with the breakage of the substituent biguanidinum group. The third stage of mass loss, which may be ascribed to the degradation of the residual cross-linked chitosan, is occurred within the range of 550–750 \(^\degree\)C. As shown in Table 2, the \( T_i \) of the Ch decreases from 253 to 177 \(^\degree\)C by biguanidilation, indicating that ChG is less thermally stable compared with chitosan. This may be due to disruption of the crystalline structure of chitosan, especially through electrostatic repulsion, as the result of the introduction of cationic guanidinium groups into chitosan. ChG-grafted PHB copolymers, irrespective of the PHB content, showed three stages of mass loss. The TGA curves for ChG-grafted PHB copolymers exhibited two stages of main thermal degradation processes. The first one (second mass loss step) that occurred in the temperature range 190–250 \(^\degree\)C with mass loss of ca. 13%, 22% and 35% for ChG-PHB1, ChG-PHB2 and ChG-PHB3, respectively, was assigned to the chain-scission of the PHB grafted chains. While the second thermal degradation (third mass loss step), occurred in the temperature range 255–310 \(^\degree\)C with mass loss of ca. 64%, 59% and 50% for ChG-PHB1, ChG-PHB2 and ChG-PHB3, respectively, may be due to the dehydration and degradation of the chitosan base polymer together with the breakage of the substituent biguanidinium group. These individual degradation processes were used to check the copolymers composition, and it was found to correlate well with the composition calculated. Compared with the thermal property of ChG (Table 2), it was found that ChG-grafted PHB samples were of higher thermal stability than ChG although the second degradation stage starts earlier in case of the copolymers. This indicates that the morphological structure of copolymers might affect the rate of degradation of ChG. Moreover, the onset degradation temperature (\( T_i \)) of the copolymers increases with increasing the content of PHB. This may be due to the introduction of PHB, which could enhance the thermal stability of the ChG, i.e. \( T_i \) for ChG-PHB1, ChG-PHB2 and ChG-PHB3, respectively.

4.2.2. Differential scanning calorimetry (DSC)
DSC scans of ChG, PHB and ChG-grafted PHB copolymers are shown in Fig. 5 and the main thermal transitions are summarized in Table 2. The first heating scan (Fig. 5a) showed broad endothermic peaks at 79, 62, 51 and 51 \(^\degree\)C with \( \Delta H = 209, 128, 58 \) and 51 \( \mathrm{J} \cdot \mathrm{g}^{-1} \) for ChG, ChG-PHB1, ChG-PHB2 and ChG-PHB3, respectively. This peak is related to the evaporation of adsorbed water due to the hygroscopic nature of ChG and its graft copolymers; the values of the enthalpy of water evaporation (\( \Delta H_{evap} \)) decreases with increasing the PHB content due to the hydrophobic nature of PHB. The PHB prepolymer exhibited a double melting endotherm (Fig. 5a), which could be ascribed to the presence of different interlamellae dimensions in the crystallites, and the differences in the microstructure of the prepolymer due to random scissions of long PHB chains [65]. With respect to the copolymers, there were no significant changes in the melting temperatures relative to that of PHB. Melt crystallization temperatures, during cooling, indicated that the crystallization rate of PHB is enhanced upon increasing its content in the copolymers (Fig. 5b and Table 2). The second heating scans of all copolymers (Fig. 5c) showed similar profiles of the melting peaks as recorded from the first heating scans, but their values are slightly shifted to higher values and \( \Delta H_m \) increased with increasing the content of PHB. This result also indicated that the crystallinity of the graft copolymers increases as the content of PHB increase.

4.3. Antimicrobial activity
4.3.1. Antibacterial activity
Antibacterial activity of Ch, ChG and ChG-grafted PHB derivatives against S. pneumoniae, B. subtilis and E. coli are collected in Table 3. In fact, chitosan inhibits bacteria by destroying the cell membrane of bacteria through electrostatic attraction between positively charged chitosan molecules and negatively charged microbial cell membrane [66] or through the penetration of the cell membrane into the nuclei of the microorganisms, which leads to the inhibition of the mRNA and protein synthesis [67]. Compared with chitosan, the biguanidinylated chitosan showed excellent antibacterial activity with low MIC value in the range of 0.49–3.90 \( \mu \)g mL\(^{-1} \) for all tested bacteria. Because the strongly basic guanidinium group could be fully protonated in the neutral
conditions, the introduction of biguanidinium group into chitosan chain could result in the increase of its cationic charges, and this is advantageous for the combination of guanidinylated chitosan with bacteria resulting in anionic charges on their surfaces. Furthermore, the trace metal cations, which are necessary for the microorganism's growth, could be selectively chelated by the biguanidinium group. All these factors could promote the guanidinylated chitosan to be associated with negatively charged substances, such as proteins, phospholipids and fatty acids, at the surface of bacteria cell and subsequently suppress their biosynthesis, disrupt the mass transport across the cell wall, and accelerate the death of the bacteria [32,66]; indeed its mechanism of biocidal action is considered to damage to the cytoplasmic membrane. For ChG-grafted PHB derivatives, ChG-PHB1 showed low activity close to chitosan, while the antibacterial activity of ChG-PHB2 was close to that of ChG. The copolymer with the high content of PHB (ChG-PHB3) showed the best activity with the lowest MIC values in the range of 0.49–0.98 μg mL⁻¹ when compared with chitosan and the prepared derivatives. Furthermore, the activity of ChG-PHB2 and ChG-PHB3 against *E. coli* was better than that of standard drug gentamicin.
with low MIC values 1.95 μg mL⁻¹ and 0.98 μg mL⁻¹, respectively, and antibacterial activity of ChG-PHB2 against *B. subtilis* was the best with MIC value 0.49 μg mL⁻¹.

Statistical analysis showed significant differences (*P* < 0.05) between the tested samples compared with chitosan (Table 3). There is insignificant difference between ChGs sample and standard Ampicillin drug against *S. pneumoniae* (*P* = 0.176). In addition, there is insignificant differences compared with standard gentamicin drug for ChG (*P* = 1.000) and ChG-PHB3 (*P* = 0.178).

### 4.3.2. Antifungal activity

The results of antifungal activity of Ch, ChG and ChG- grafted PHB derivatives against *A. fumigatus*, *G. candidum* and *S. recemosum* fungi are given in Table 4. Compared with the antifungal activity of Ch, ChG has better activity with MIC values in the range of 0.49–3.9 μg mL⁻¹ for all tested fungi due to the introduction of biguanidinium groups. On the other hand, the ChG-PHB2 and ChG-PHB3 has better antifungal activity than ChG-PHB1, but lower activity than ChG. Furthermore, all the samples showed antifungal activity in the order *S. recemosum* > *A. fumigatus* > *G. candidum*.

Statistical analysis showed there were significant differences between tested samples compared with chitosan (Table 4). There is insignificant difference between ChG sample and standard amphotericin B drug against *A. fumigatus* and *S. recemosum* (*P* = 0.147 and 0.066, respectively). Also there is insignificant difference between ChG-PHB3 sample and standard amphotericin B drug against *S. recemosum* (*P* = 0.318)

### 5. Conclusions

Graft copolymers based on chitosan biguanidinium hydrochloride (ChG) and poly[(R)-3-hydroxy butyrate] (PHB) were successfully synthesized, via condensation reaction of the carboxylic groups of PHB prepolymer with the amino groups of ChG, and characterized by means of FTIR, 1H NMR, XRD, TGA and DSC analyses. XRD data showed that, the ChG- grafted PHB copolymers exhibit somewhat a crystalline fraction. TGA results showed that ChG- grafted PHB samples are less thermally stable than chitosan, but of higher thermal stability than ChG. Moreover, the onset degradation temperature (*T*<sub>d</sub>) of the graft copolymers is found to increase with increasing the extent of PHB. This may be due to the introduction of PHB, which enhances the thermal stability of the ChG. DSC data showed that, the enthalpy of water evaporation of copolymers decreases with increasing the content of PHB as a result of the hydrophobic character of PHB. Moreover, melt crystallization temperatures revealed that the crystallization rate of PHB is enhanced with increasing its content in the copolymers.

Antimicrobial activity for chitosan, ChG and ChG- grafted PHB copolymers was studied against *Streptococcus pneumoniae*, *Bacillus subtilis*, *Escherichia coli* (as examples of bacteria) and *Aspergillus fumigatus*, *Geotricum candidum* and *Syncephalastrum recemosum* (as examples of fungi). The results indicated that the prepared chitosan derivatives show excellent antimicrobial activity. Moreover, the antibacterial activity of ChG-PHB2 and ChG-PHB3 against *E. coli* is better than that of ChG, while ChG-PHB3 has the best antibacterial
activity against B. subtilis. These copolymers are of great importance because of their potential applications in biodegradable and biomedical materials.

References