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ORIGINAL PAPER

Biodegradable and biocompatible radiopaque iodinated poly-3-hydroxy butyrate: synthesis, characterization and in vitro/in vivo X-ray visibility

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Abstract

Some novel radiopaque biodegradable and biocompatible iodinated polymers based on poly-3-hydroxy butyrate (PHB) were obtained. Following the attachment of diethanol amine to PHB, the hydroxyl ends were capped with 4-iodobenzoic acid and 2,3,5-tri-iodobenzoic acid. In this manner, tri-novel radiopaque polymers were obtained. The resulting polymers were structurally characterized by NMR technique. They were evaluated with respect to their possible use as radiopaque implant biomaterials indicating X-ray visibility in a noninvasive manner using routine X-ray absorption imaging techniques. These polymers exhibited good radiopacity with conventional imaging X-ray techniques in vivo. Additionally, biocompatibility of these iodinated polymers was also evaluated. There were no signs of infection or abscess formation on the surgical area. These novel radiopaque PHBs should be promising biomaterials for a new-generation radiopaque materials.

Keywords Poly-3-hydroxy butyrate (PHB) · Radiopaque · Biocompatibility · In vivo experiment

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Introduction

Poly(3-hydroxy alkanoate)s (PHAs) are storage materials for some microorganisms. They are hydrophobic biodegradable polyesters that their structures can be controlled by the carbon source [1–5]. PHAs are generally classified in two types: short-chain-length (scl) and medium-chain-length (mcl) PHAs. Both types of the PHAs need modification reactions [6–9] in order to improve hydrophilicity and mechanical properties [10–17]. Because of their biodegradability and biocompatibility, PHAs are potential candidates for medical applications [18–22]. Poly-3-hydroxy butyrate (PHB) is a member of PHAs. PHB is a crystalline, brittle polymer with high melting transition temperature (~ 170 °C). There are few chemical modification reactions of PHB. Functionalization of PHB was carried out by chlorination in our laboratories [23, 24]. The chlorinated PHB was blended with polymethyl methacrylate in view of its optical behavior [25]. Modified PHB can also be obtained by the anionic polymerization of α-methyl-α-pentyl-β-propiolactone [26]. Radiopaque materials absorb X-rays; therefore, they can be visualized and traced in the human body. There have been many attempts to modify the frequently used medical products to enhance visibility. In this manner, X-ray visible $BaSO_4$ nanoparticles were used in trans-catheter arterial embolization procedures [27] and in bone contact [28]. In order to enhance radiopaque properties of the polymers, monomers containing heavy halogen atoms such as bromine and iodine were introduced. Iodine has been widely used to enhance radiopacity because of its great mass attenuation and documented low systemic toxicity [29–32]. Meng et al. reported the preparation and evaluation of radiopaque microspheres based on polyvinyl alcohol and lipiodol which is poppy seed oil that contains 38% iodine by weight [33]. Iodinated radiopaque methacrylate copolymer can be prepared via the ring-opening polymerization of glycidyl methacrylate [34]. van Hooy-Corstjens et al. reported the iodinated methacrylic polymer cages to restore the height between two adjacent vertebrae [35]. The iodine-containing methacrylic copolymers were also used into the cement powder [36]. Estep et al. synthesized novel radiopaque oils, the 1,3,5-trialkyl-2,4,6-triiodobenzenes, for mucosal coating in the gastrointestinal tract [37]. Koole and co-workers prepared monomers containing covalently bonded iodine and copolymerized with 2-hydroxyethyl methacrylate and methyl methacrylate for x-ray visible stent application indicating high stability in living body [38, 39]. Herein, we report first time the synthesis and characterization of novel radiopaque PHB derivatives. PHB is renewable, biodegradable and biocompatible microbial polyester. It is also commercially available. To gain radiopacity to this very valuable biomaterial is very important for the medical applications in vivo. The carboxylic acid end was reacted with diethanol amine in order to obtain PHB with three hydroxyl groups. Then, the hydroxyl groups were reacted with iodobenzoic acid derivatives in order to obtain novel biodegradable radiopaque biodegradable polymers. Structural characterization was performed by proton, carbon NMR and size exclusion chromatography. X-ray visibility of the obtained polymers was confirmed in vivo and in vitro.

Experimental

Materials

Poly-3-hydroxy butyrate (PHB), microbial polyester, was supplied from BIOMER (Germany). Dimethyl formamide (DMF), *N*,*N*′-dicyclohexylcarbodiimid (DCC), dimethyl amino pyridine (DMAP), stannous 2-ethyl hexanoate (Sn-oct), diethanol amine (DEA), 4-iodobenzoic acid (IB), 4-iodobenzoyl chloride (IB-Cl), triethyl amine (TEA), 2,3,5-tri-iodobenzoic acid (3IB) and the other chemicals were purchased from Sigma-Aldrich and used without further purification.

Synthesis of hydroxylated PHB (PHB-DEA)

Hydroxylated PHB was obtained by the reaction of PHB with DEA according to the procedure reported in cited Ref. [40].

The modified procedure is as follows: A mixture of 60.3 g of vacuum-dried PHB, 102.4 g of DEA and 2.1 g of Sn-oct in 250 mL of $CHCl_3$ was stirred at room temperature for 24 h. Then, it was refluxed for 3 h. The solvent was distilled under atmospheric condition (not in the rotary evaporator). Then, the crude product was cured in 125 °C for 1.5 h. The product was washed with excess methanol and filtered. The crude product was dried under vacuum at 40 °C for 24 h. For further purification, the obtained polymer was dissolved in 600 mL of $CHCl_3$ and filtered. The solvent was evaporated in a rotary evaporator. The obtained polymer was washed with excess methanol again. The pure diethanol amine derivative of PHB, three hydroxyl-terminated PHB, was filtered and dried under vacuum 40 °C for 24 h. Yield was 56 g. This was coded as PHB-DEA. Characteristic FTIR signals: 1567 cm^{-1} amide carbonyl; 3301 cm^{-1} primary hydroxyl groups of DEA; 1721 cm^{-1} belong to ester carbonyl of PHB. The characteristic chemical shifts of the PHB-DEA sample in ^1H NMR spectrum were observed at 1.3 ppm for $–CH_3$, 2.4–2.6 ppm for $–CH_2–COO–$, 3.0 ppm for $–N–CH_2–$, 3.5–3.8 ppm for $–CH_2–OH$, 4.1 ppm for –CH–OH and 5.1–5.3 ppm for –CH–O– [40]. The GPC result was M_n 32,813 Da, M_w 47,700 Da, PDI 1.45; M_n 7800 Da, M_w 8706 Da, $Đ$ 1.12.

Synthesis of iodinated PHBs

The iodinated PHB samples (PHB-DEA-IB-6, PHB-DEA-IB-12 and PHB-DEA-3IB-1) were obtained by the reaction between PHB-DEA and IB/3IB according to the Steglich esterification method [41].

PHB-DEA-IB-6

PHB-DEA (9.8 g) was dissolved in a mixture of CH_2Cl_2 (100 mL) and DMF (5 mL). To this solution were added under continuously stirring 4-iodobenzoic acid (6.3 g, 0.025 mol), DCC (9.2 g, 0.045 mol) and DMAP (0.81 g, 0.0066 mol) under argon.

Yield was 10.7 g. After stirred at room temperature for 24 h, the precipitated side product, dihexyl urea, was filtered. The solvent of the filtered solution was evaporated, the crude product was leached with excess methanol, and the iodinated PHB, PHB-DEA-IB-6, was purified via filtering of methanol. The product was dried under vacuum at 40 °C for 24 h. The GPC result was M_n 29,100 Da, M_w 38,500 Da, $Đ$ 1.33.

PHB-DEA-3IB-1

The same procedure was repeated using the following reagents: PHB-DEA (4.85 g) was dissolved in a mixture of CH_2Cl_2 (50 mL) and DMF (5.0 mL). To this solution were added under continuously stirring 2,3,5-tri-iodobenzoic acid (3.42 g, 0.0068 mol), 50 mL of 1 M DCC in CH_2Cl_2 (0.050 mol) and DMAP (1.32 g, 0.010 mol) under argon. Yield was 5.78 g. The GPC result was M_n 30,800 Da, M_w 40,000 Da, $Đ$ 1.30.

PHB-DEA-IB-1

PHB-DEA-IB-1 was obtained by the reaction between hydroxylated PHB and 4-iodobenzoyl chloride in conventional manner. Briefly, PHB-DEA (2.16 g) was dissolved in a mixture of CH_2Cl_2 (30 mL) and triethylamine (0.42 g, 0.004 mol). The solution was chilled under 10 °C using ice/water mixture. 4-Iodobenzoyl chloride (3.42 g) in 10 mL of CH_2Cl_2 was added into the cold solution with continuously stirring in 5 min. The solution was left to warm up to room temperature for overnight. The needle crystals of the triethylamine hydrochloride side product were removed via filtration process. The solvent was evaporated and precipitated with cold petroleum ether (100 mL). The product, PHB-DEA-IB-1, was dried under vacuum at 40 °C for 24 h. Yield was 2.88 g.

PHB-DEA-IB-3

PHB-DEA (1.75 g) was dissolved in a mixture of CH_2Cl_2 (30 mL) and triethylamine (0.50 g, 0.005 mol). To this solution was added solid 4-iodobenzoyl chloride (0.81 g) with continuously stirring in 5 min. The solution was continuously stirred at room temperature for 24 h. The needle crystals of the triethylamine hydrochloride side product were removed via filtration process. The solvent was

evaporated and precipitated with cold petroleum ether (100 mL). The product, PHB-DEA-IB-3, was dried under vacuum at 40 °C for 24 h. Yield was 0.87 g.

Characterization of the polymers synthesized

Molecular weights were determined by size exclusion chromatography instrument, Viscotek GPCmax Auto sampler system, consisting of a pump, three ViscoGEL GPC columns (G2000H HR, G3000H HR and G4000H HR) and a Viscotek differential refractive index (RI) detector with a THF flow rate of 1.0 mL/min at 30 °C. A calibration curve was generated with three polystyrene (PS) green standards: 2960, 50,400 and 696,500 Da, of low polydispersity. The polymer sample solution containing 0.05 g in 10 mL of THF was filtered and injected automatically into the instrument. Data were analyzed using Viscotek Omni SEC Omni 01 software.

Proton NMR spectra in $CDCl_3$ solutions of the samples were taken at a temperature of 25 °C with an Agilent NMR 600-MHz NMR (Agilent, Santa Clara, CA, USA) spectrometer equipped with a 3-mm broadband probe.

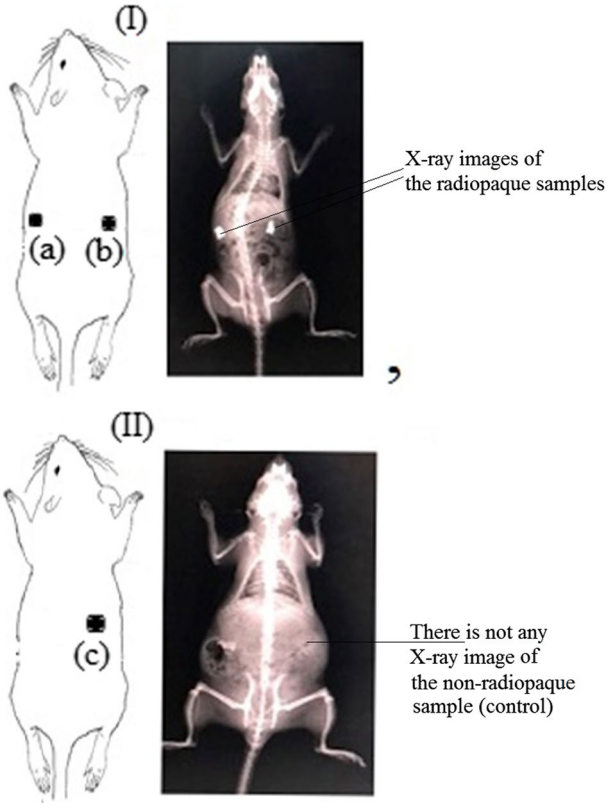


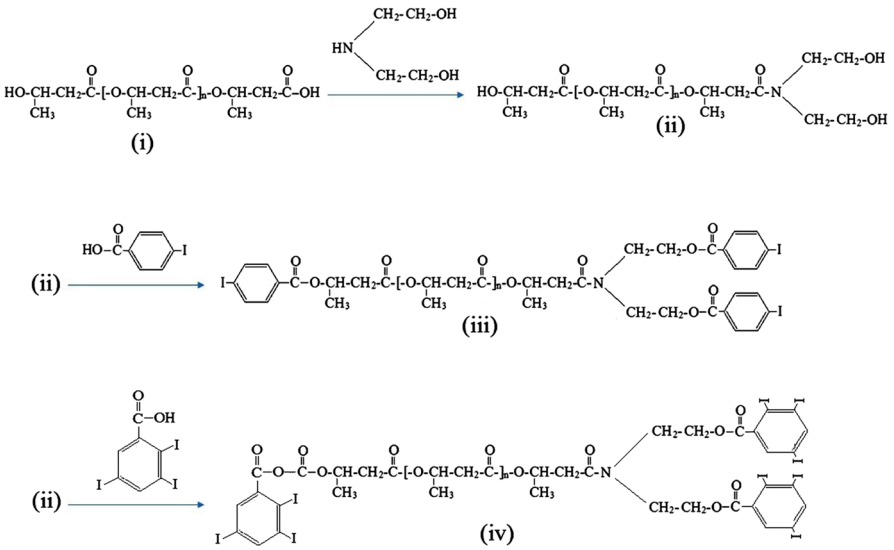
Fig. 1 X-ray images of rats: in rat I, **a** PHB-DEA-IB-6, **b** PHB-DEA-3IB-1; in rat II, **c** PHB-DEA (control)

In vivo implantation

Two female albino Wistar rats with an average weight of 230 g were used in this study. The animals were housed and fed adlibitium and divided into two: rat I and rat II. All rats were anesthetized with intraperitoneal injection of a 0.1 mL/kg alphazyn and 0.3 mL/kg ketamine mixture. Similar to our previous studies [42–44], the biomaterial samples in sizes approximately 0.5 × 0.5 cm and 1.9 mm thickness were placed symmetrically as shown in Fig. 1 under the skin of the back of the rat, on top of the muscle fascia. In rat I, two different iodinated polymer samples were implanted: PHB-DEA-IB-6 (a), PHB-DEA-3IB-1 (b) and (c) PHB-DEA (control).

In vivo biocompatibility–X-ray studies of the PHAs

On the fifth day of the implantation, the rats were sedated with the same protocol as above, and X-ray images of the surgical area were taken by a conventional X-ray device. (Siemens, Luminos dRF Max, X-ray generator 65 kW). On the same day, the rats were killed. There were no signs of infection or abscess formation on the surgical area. Our study was approved by Animal Research Ethics Committee of Bezmialem Vakıf University Experimental Research Center (approval number; 2018/10).



Scheme 1 Synthesis of iodobenzoyl derivatives of PHB. **i** PHB, precursor, **ii** PHB-DEA, **iii** PHB-DEA-IB (PHB-DEA-IB-1, PHB-DEA-IB-3, PHB-DEA-IB-6, PHB-DEA-IB-12), **iv** PHB-DEA-3IB-1

Results and discussion

Iodinated PHB samples

PHB is microbial polyester with one hydroxyl and one carboxylic acid end. Diethanol amine was capped with carboxylic end of PHB to obtain hydroxylated PHB with three hydroxyl ends. As precursor, tri-hydroxylated PHB (PHB-DEA) was synthesized more than twenty times. The obtained PHB-DEA samples were structurally characterized by the NMR technique. All samples indicated the characteristic signals. Reaction pathways of the iodinated PHB samples are shown in Scheme 1.

In order to evaluate the esterification syntheses, Steglich esterification and esterification with acid chloride methods were used to obtain iodinated PHB radiopaque derivatives. Some different molar ratios of the reagents resulted in iodinated products. Results and conditions of the iodinated PHB derivatives are shown in Table 1.

The hydroxyl ends of the PHB-DEA were reacted with 4-iodo- and 2,3,5-tri-iodobenzoic acids to obtain radiopaque iodinated PHB derivatives. The obtained polymers were purified by precipitating in excess methanol at least in three times. So, unreacted iodobenzoic acid starting materials were removed from the obtained product. Molar masses of the purified iodinated PHB derivatives were at around 30,000 g/mol (Mn) with polydispersity 1.30. The iodobenzene derivatives of PHB were characterized using proton and carbon NMR techniques. Figure 2 shows ^{1}H NMR spectra of the 4-iodobenzene and 2,3,5-tri-iodobenzene derivatives of PHB: PHB: PHB-DEA-IB-6 and PHB-DEA-3IB-1, respectively. Typical chemical shifts of the iodobenzoyl protons have been localized in between 7.6 and 7.8 ppm while the tri-iodobenzoyl protons were localized in chemical shifts 8.3 and 7.75 ppm.

^{13}C NMR spectrum of PHB-DEA-IB-6 is shown in Fig. 3. The characteristic iodobenzoyl groups of the iodinated PHB were observed in their ^{13}C NMR spectra. The chemical shifts at 139, 132, 128 and 101 ppm (a, b, c and e, respectively) are assigned to the ring carbons [45]. As for tri-iodobenzoyl derivative of PHB, ^{13}C NMR spectrum of PHB-DEA-3IB-1 contained the characteristic signals related to the ring carbons, as well. As shown in Fig. 4, the signals/ppm at 149, 145, 137, 114, 106 and 94 ppm were assigned to the ring carbons.

In vitro X-ray analysis of the PHB derivatives

The X-ray visibility of the iodinated PHB derivatives was studied under X-ray irradiation. The images of the PHB samples were taken under daylight and under X-ray irradiation. Figure 5 shows the images of the iodinated PHB derivatives: PHB-DEA-IB-6 (a-1) under daylight, (a-2) under X-ray irradiation; PHB-DEA-3IB-1 (b-1) under daylight, (b-2) under X-ray irradiation; PHB-DEA-IB-1 (c-1) under daylight, (c-2) under X-ray irradiation. Iodobenzoyl derivatives of PHB (a,

Table 1 Reaction conditions, synthesis and characterization of the iodinated PHB derivatives

Code	PHB-DEA (g)	IB		3IB		DCC		DMAP		IB-Cl		TEA		Yield (g)
		(g)	(mmol)	(g)	(mmol)	(g)	(mmol)	(g)	(mmol)	(g)	(mmol)	(g)	(mmol)	
PHB-DEA-IB-6	9.80	6.30	25	–	–	9.20	45	0.81	7.0	–		–		10.7
PHB-DEA-3IB-1	4.85	–	–	3.42	7.0	1.12	50	1.32	10	–				5.78
PHB-DEA-IB-1	2.16	–	–	–	–	–	–	–	–	3.42	13	0.42	4.0	2.88

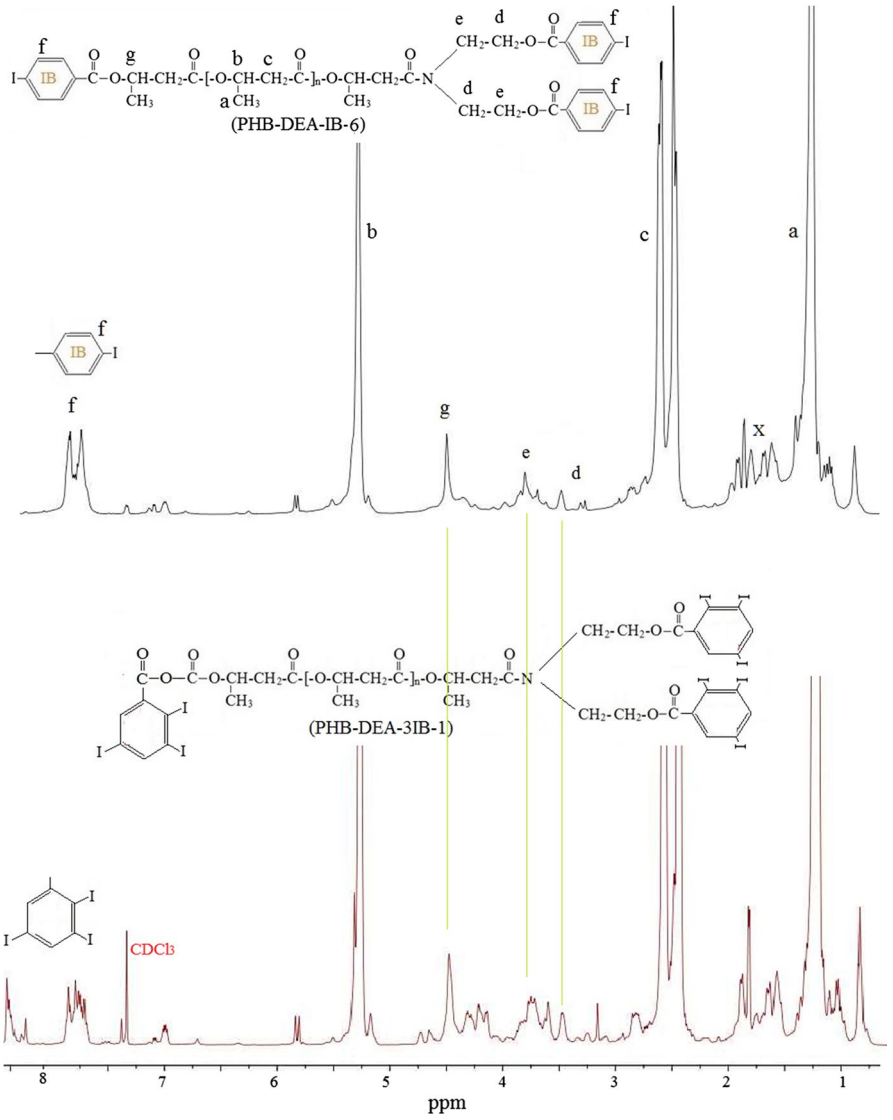


Fig. 2 ^{1}H NMR spectra of the 4-iodobenzene and 2,3,5-tri-iodobenzene derivatives of PHB:PHB:PHB-DEA-IB-6 and PHB-DEA-3IB-1, respectively

b, c) all showed the X-ray visibility while PHB without iodine content did not show visibility. Interestingly, 4-iodobenzoate derivative of PHB (PHB-DEA-IB-6 and PHB-DEA-IB-1) showed the higher radiopacity than the tri-iodo derivative of PHB (PHB-DEA-3IB-1). Maybe, the less thickness of the rectangular mold affects of the opacity.

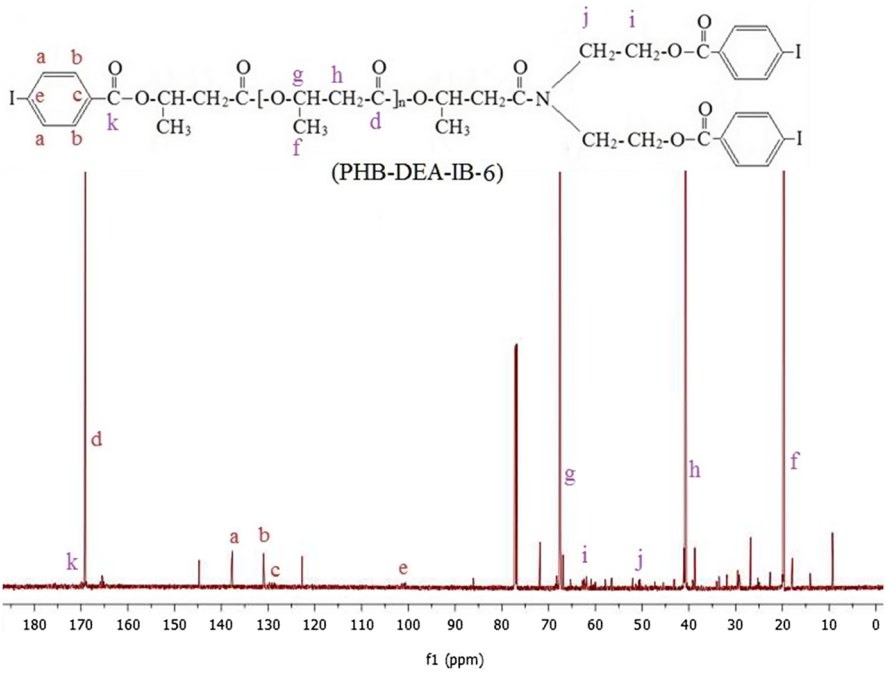


Fig. 3 ^{13}C NMR spectrum of the iodinated PHB sample containing 4-iodobenzoyl groups (PHB-DEA-IB-6)

Biocompatibility of the novel polymers

Biocompatibility of implanted radiopaque microspheres was investigated in vivo (Fig. 6). The implanted samples were found to be well tolerated. There were no signs of infection or abscess formation on the surgical area.

X-ray analysis

In rat I, the polymer stated as control group (PHB-DEA-22 on left side) was not visualized under the X- ray as expected. All the other polymer samples implanted revealed radiopacity under the X-ray. Polymers implanted to the rat II (PHB-DEA-IB-6 on left side- PHB-DEA-3IB-1 on right side) seem to have a higher opacity than the rat I polymers. Additionally, the polymer sample PHB-DEA-IB-12 implanted to the rat I seem to have similar density of radiopacity with the vertebral bone of the rat, whereas the polymer samples implanted to the rat II reveal higher radiopacity compared to the skeleton of the rat.

PHB, a well-known biocompatible polymer, has wide variety of clinical applications. It has been used as micelles, hydrogels and micro-carrying system for drugs, and chemotherapeutics. In some cases, clinicians might need to trace the carrying system in vivo to see the therapeutic effect of the drug or the agents. There are ways to trace the carrying systems: adding a fluorescent property to the hydrogels

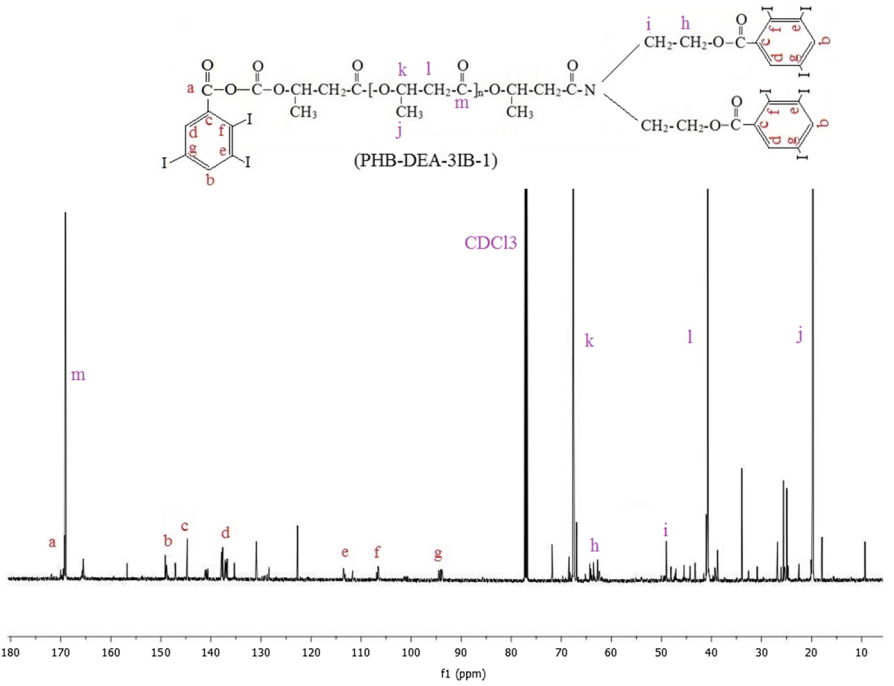


Fig. 4 ^{13}C NMR spectrum of the iodinated PHB sample containing 2,3,5-tri-iodobenzoyl groups (PHB-DEA-3IB-1)

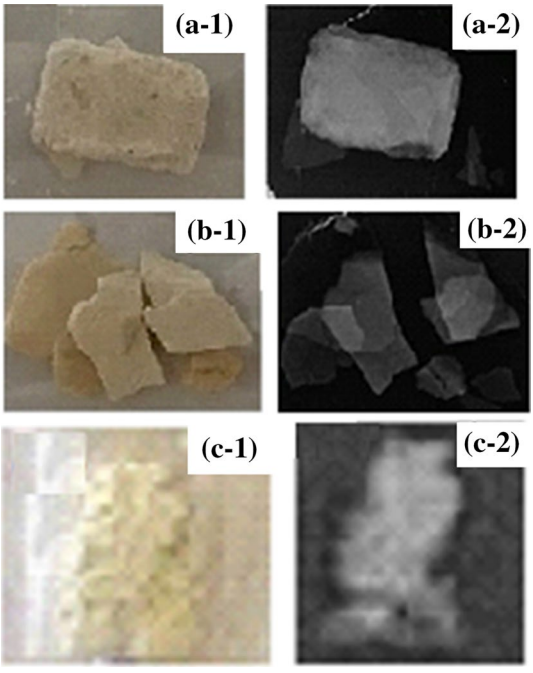


Fig. 5 Images of the iodinated PHB derivatives: PHB-DEA-IB-6 **a-1** under daylight, **a-2** under X-ray irradiation; PHB-DEA-3IB-1 **b-1** under daylight, **b-2** under X-ray irradiation; PHB-DEA-IB-1 **c-1** under daylight, **c-2** under X-ray irradiation

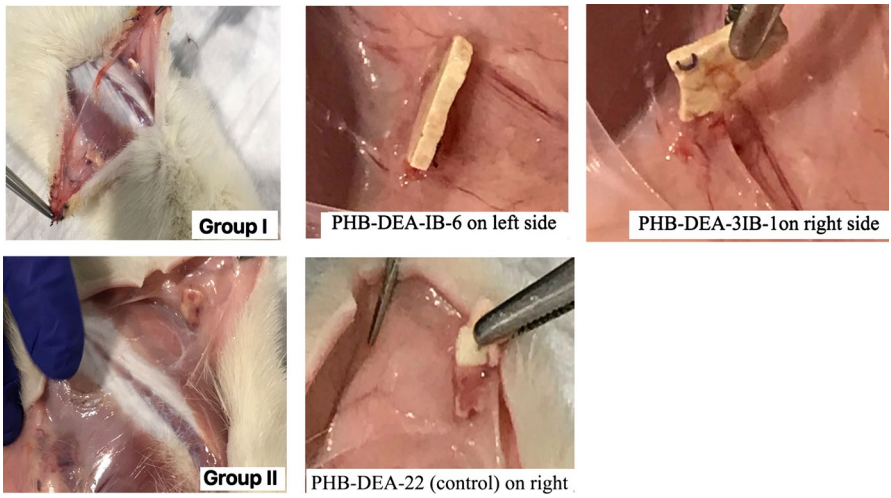


Fig. 6 In vivo appearance–biocompatibility assessment of the polymers after 5 days of implantation under the skin of the rats is seen

or micelles or adding heavy metals such as Fe to be detected in magnetic resonance images in blood samples [46].

However, the most practical way to trace a polymer in vivo is with its radiopacity. If this material is visible under an X-ray image, then the material can be seen in any part of the body. Additionally, the quantity of the material can be detected with the degree of enhancement of the material in an X-ray image. In the literature, shape memory polyutherane foams were presented with enhanced radiopacity, which was used as filling in intracranial aneurysm treatment. These polyutherane foams seem to be visible through the skull also [47]. In our study, we have modified three hydroxylated PHB with iodobenzoic acid derivatives in order to add radiopacity. We could see that we can adapt the radiopacity of the polymer with the modification of the polymer.

With this radiopacity modification, these biopolymers can be use in clinical applications as a substitute for muscle facias in abdominal surgery, tendon substitutes or as a dura in spinal surgeries. This property will increase the clinical application diversity of the biomaterial.

Conclusion

These novel radiopaque polymeric materials have a potential as new bulking agents for radiological observation. Its advantage over the existing bulking agents lies in their X-ray visibility in situ. These novel polymers were also found to be biocompatible. This is encouraging with respect to the intended application, although it must be acknowledged that the biocompatibility studies should be performed with longer period of time resembling more of clinical application. PHB is a very unique

biodegradable, biocompatible, microbial polyester obtained from ubiquitous renewable resources such as CO_2, sugar, acetic acid and plant oils. Radiopaque PHB could be a very special biomaterial for biotechnology. To gain radiopacity to this very valuable biomaterial is very important for the medical applications in vivo. The first time obtained novel radiopaque PHB is the first example of the very large microbial polyester family. We believe that this first time synthesized novel radiopaque PHB will be very attractive and a promising biomaterial in tissue engineering and drug delivery systems.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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