

Effects of sterilisation by high-energy radiation on biomedical poly-(ϵ -caprolactone)/hydroxyapatite composites

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Abstract The effects of a high energy sterilization treatment on poly- ϵ -caprolactone/carbonated hydroxyapatite composites have been investigated. Poly- ϵ -caprolactone is a biodegradable polymer used as long-term bioresorbable scaffold for bone tissue engineering and carbonated hydroxyapatite is a bioactive material able to promote bone growth. The composites were gamma-irradiated in air or under nitrogen atmosphere with doses ranging from 10 to 50 kGy (i.e. to a value higher than that recommended for sterilization). The effects of the irradiation treatment were evaluated by vibrational spectroscopy (IR and Raman spectroscopies) coupled to thermal analysis (Differential Scanning Calorimetry and Thermogravimetry) and Electron Paramagnetic Resonance spectroscopy. Irradiation with the doses required for sterilization induced acceptable structural changes and damaging effects: only a very slight fragmentation of the polymeric chains and some defects in the inorganic component were observed. Moreover, the radiation sensitivity of the composites proved almost the same under the two different atmospheres.

1 Introduction

Tissue engineering is a new emerging interdisciplinary field of biomaterial science aimed at overcoming the lack of donors for tissue replacements. One of the most recent tasks is to create a porous template, called scaffold, to replace human bone tissue. Poly(ϵ -caprolactone) (PCL) is a biodegradable aliphatic poly(α -hydroxy acid) widely used in biomedical applications, due to its biocompatibility and bioresorbability (Fig. 1). PCL-based products can be used in bone tissue engineering since they possess adequate mechanical properties to serve as scaffolds for the period required by bone to heal (6 months at least) [1–4]. In fact, the three-dimensional synthetic scaffolds, implanted at the tissue defect site, provide a framework for cells to attach, proliferate and form extra-cellular matrix. To promote the bone regeneration process, carbonated hydroxyapatite (HA), the main component of the inorganic phase of bone, can be added to the polymeric material (Fig. 1).

The host response to the implanted biomaterial depends on numerous factors, including sterilisation. Several procedures (e.g. steam, ethylene oxide gas, low temperature plasma, dry heat, high-energy irradiation) have been developed to sterilise medical devices and long-term implants [5, 6], but only few of them are appropriate for short-term implants such as PCL-based materials. Since they are sensitive to both dry heat sterilisation and steam sterilisation, high-energy irradiation is likely to be the method of choice for many PCL implants. Being highly penetrative, gamma radiation allows to uniformly sterilize scaffolds (which are often characterized by a high porosity) without leaving residues which can affect the biological performance of the implants [7].

The doses for radiation sterilisation of medical devices and health care products, as well as the validation

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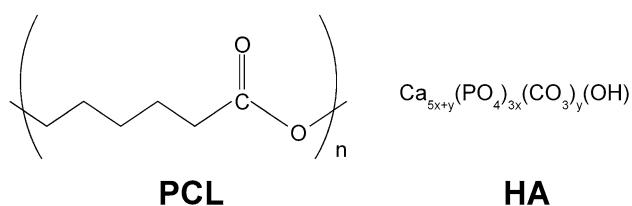


Fig. 1 Chemical formula of poly(ϵ -caprolactone) (PCL) and carbonated hydroxyapatite (HA)

procedures, are defined in standard ISO 11137. For tissue allografts, the international tissue bank standard recommends 15 kGy as a minimum dose for bacteria decontamination and 25 kGy as minimum dose for bacteria sterilisation [8, 9], while other international tissue banks recommend doses up to 35 kGy [10].

However, the high-energy radiation treatment, in addition to the destruction of bacteria, may also affect the chemico-physical properties of the material [11–16]. Upon irradiation, free radicals are formed in the excited polymeric material and chemical changes can be induced. As a consequence, cross-linking reactions or shortening of the chain length can occur, depending on the radiation dose and environment [17, 18]. In general, aromatic polymers are more resistant to sterilisation by high-energy radiation than aliphatic polymers, while the presence of impurities and additives may enhance degradation and/or crosslinking [19, 20].

The radiation-induced changes may also lead to modifications in degradation rate if compared to non-sterilised materials. In other words, gamma irradiation cannot be considered per se as detrimental, since a property enhancement rather than a loss of property could be achieved depending on the radiation dose and environment.

In this light, the present study aims at evaluating the possible effects induced by gamma irradiation on both the inorganic and polymeric components of some PCL/HA composites; these effects could be used in a controlled manner to introduce positive changes in the material. To this purpose, we exposed PCL/HA samples with different PCL/HA ratios to gamma radiation under nitrogen atmosphere or in air. Different doses were administered, up to 50 kGy (i.e. a dose greater than that required for sterilisation) [21].

To evaluate the possible effects of the high-energy radiation treatment, the samples were analysed by vibrational spectroscopy (IR and Raman spectroscopies) coupled to thermal analysis (Differential Scanning Calorimetry and Thermogravimetry), which are highly sensitive to structure variations [22–25]. Electron Paramagnetic Resonance (EPR) spectra of the irradiated samples were also recorded to evaluate possible defects in the inorganic part of the composites.

2 Materials and methods

2.1 Composites

The PCL/HA composites (4 mm \times 4 mm \times 2 mm) were synthesised at the Institute of Composite and Biomedical Materials, (National Research Council, Naples, Italy) using phase-inversion and a salt-leaching technique, as described elsewhere [26]. The samples, containing different PCL/HA (w/w) ratios, were labelled as indicated in Table 1. Control samples of pure PCL and HA were also analysed. The surface morphology of the pure PCL and the composites before irradiation was checked by scanning electron microscopy technique (SEM), resulting to be similar to those just reported in the literature [24].

2.2 Gamma-irradiation

The samples were irradiated at ISOF (Institute for the Organic Synthesis and Photoreactivity of the National Research Council of Italy) with gamma rays from a ^{60}Co Nordion 220 Gammacell (Fig. S1). The gammacell was calibrated using an alanine-EPR transfer dosimetry system traceable to Risø High Dose Reference Laboratory and Fricke chemical dosimeter. Irradiation was performed for a maximum of 77 h. The dose rate at the sample location was 0.65 kGy/h with an uncertainty of $\pm 2\%$ (1 SD). Samples were inserted in a poly-methyl-methacrylate holder (5 mm thick walls suitable for electronic equilibrium and 5 cm internal diameter) and three doses were administered (from 10 up to 50 kGy, dose to water).

The samples were irradiated either under nitrogen into sealed vials or in air to evaluate the influence of the atmosphere on material properties. This aspect can be important for the global cost of industrial sterilisation treatments often requiring the use of inert atmosphere to minimise the formation of oxygen radical species.

Table 1 Calorimetric data obtained for the untreated PCL/HA scaffolds by different techniques

| Sample ID | PCL% w/w ^a | Crystallinity degree ($X_c\%$) ^b | Crystallisable fraction (CF%) ^b |
|--------------|-----------------------|---|--|
| PCL | 100 | 73 | 69 |
| PCL/HA 70/30 | 73 | 75 | 64 |
| PCL/HA 60/40 | 62 | 76 | 60 |
| PCL/HA 50/50 | 52 | 77 | 59 |

^a Data obtained by TG measurements

^b Data obtained by DSC measurements coupled to TG

2.3 TG and DSC analysis

Thermogravimetry (TG) measurements of the samples were performed with a Mettler TA-STAR, TGA/SDTA 851° thermobalance in air, with a heating rate of 2°C/min, from 25 to 900°C: in this temperature range, the polymeric component burns (between 300 and 450°C), leaving unaltered the inorganic part.

Differential Scanning Calorimetry (DSC) measurements were performed with a Mettler TA-STAR, DSC 821° calorimeter, in the interval 5 to 120°C. The samples were heated at 2°C/min (1st run) and then cooled at the same rate down to 5°C (2nd run). Typically, for a crystalline polymer the 1st run presents the melting peak of the polymer. From this peak, the crystallinity degree ($X_c\%$) of the polymer was calculated according to the following equation:

$$X_c\% = 100(\Delta H_{m1}/\Delta H_m^\circ) \quad (1)$$

where ΔH_{m1} is the enthalpy of melting measured in the 1st run and ΔH_m° the enthalpy of melting of a totally crystalline PCL ($\Delta H_m^\circ = 139 \text{ J/g}$) [27]. ΔH_{m1} was calculated by taking into account the weight percentages of the PCL component determined by TG analysis. Only the samples irradiated at 50 kGy were analysed by these thermal techniques, due to the destructive nature of the measurements.

The crystallisation capability of the polymer, once melted in the 1st run, is evaluated as crystallisable fraction (CF%), which was calculated according to the following equation:

$$CF\% = 100(\Delta H_c/\Delta H_{m1}) \quad (2)$$

where ΔH_c is the enthalpy of crystallisation measured in the 2nd run.

2.4 Vibrational and EPR spectroscopies

Raman spectra were recorded with a Jasco R1100 spectrometer at a scattering angle of 90° with a spectral resolution of 4 cm⁻¹ using the 514.5 nm line (Innova Coherent 70) with a power of ca. 200 mW. Each spectrum was the average of eight scans.

IR spectra were recorded on a Nicolet 5700 FTIR spectrometer with a spectral resolution of 4 cm⁻¹, equipped with a diamond attenuated total reflectance (ATR) accessory and a DTGS detector. Each spectrum was the average of 200 scans.

Electron Paramagnetic Resonance (EPR) spectra were recorded at room temperature using an upgraded Bruker ER200D/ESP300 EPR spectrometer operating in the X-band. The spectrometer was equipped with a dedicated data system for the acquisition and manipulation of the

spectra, a standard variable temperature accessory, an NMR Gaussmeter for the calibration of the magnetic field, and a frequency counter for the determination of g -factors that were corrected with respect to that of the perylene radical cation in concentrated sulphuric acid. Each spectrum was the sum of four scans.

3 Results and discussion

3.1 Untreated samples

3.1.1 Thermal analysis

The data obtained from the TG and DSC thermograms of all the investigated non-irradiated samples are collected in Table 1. The TG data indicated that the percentage of the PCL component in the three different composites ranged from 52 to 73% weight/weight (w/w). DSC analysis showed that the method of synthesis used for preparing the composite has slightly modified the polymer, causing an increase in $X_c\%$ (from 73 to 77%). The crystallisable fraction percentage (CF%) of the samples showed an opposite trend, decreasing from 69 to ~60% when the HA content was $\geq 40\%$ w/w. This behaviour indicated that the presence of a certain amount of HA made crystallisation more difficult, probably because of the presence of more restrictions in the system (i.e. formation of more crystallites and, as a consequence, a higher number of inter-crystalline bonds) [28].

3.1.2 Vibrational analysis

The analysis of the IR spectra confirmed the thermal data and strengthened the idea that the polymeric component had the highest crystallinity degree in PCL/HA 50/50 composite, according to the DSC data reported in Table 1. In fact, in the spectrum of the latter composite, the bands at 1735 cm⁻¹ (ν C=O) and 1165 cm⁻¹ (ν C–O–C), typical of crystalline PCL [22, 23], showed the lowest full-width at half maximum (FWHM) (see Table 2, Fig. 2). Since PCL is a typical semi-crystalline polymer, band narrowing indicates a reduction of the amorphous domains (and a corresponding increase of the crystalline domains), due to the decrease in the dynamical disorder of the groups.

The IR bands gave information also on the apatitic component. The IR bands at ~1000 cm⁻¹ and in the 500–600 cm⁻¹ region, attributable to the PO₄³⁻ modes of HA [22, 23, 29, 30], showed their maximum intensity relatively to polymer bands in the PCL/HA 50/50 composite (Fig. 2d), according to its highest PCL/HA ratio (Table 1). In addition, the bands at 1458–1415 and 875 cm⁻¹, visible in the IR spectrum of the HA component used for preparing

Table 2 Full-width at half maximum (FWHM) of the IR marker bands of crystalline PCL of untreated and irradiated samples at 50 kGy in air

| Sample ID | Dose (kGy) | FWHM (cm ⁻¹) | |
|--------------|------------|-----------------------------------|-----------------------------------|
| | | Band at 1721 ± 1 cm ⁻¹ | Band at 1165 ± 1 cm ⁻¹ |
| PCL | 0 | 39 | 57 |
| PCL/HA 70/30 | 0 | 34 | 48 |
| PCL/HA 60/40 | 0 | 33 | 47 |
| PCL/HA 50/50 | 0 | 31 | 43 |
| PCL | 50 | 40 (40) | 57 (56) |
| PCL/HA 70/30 | 50 | 34 (35) | 47 (48) |
| PCL/HA 60/40 | 50 | 33 (32) | 46 (46) |
| PCL/HA 50/50 | 50 | 31 (31) | 43 (43) |

The corresponding values obtained after irradiation in N₂ are given into parentheses

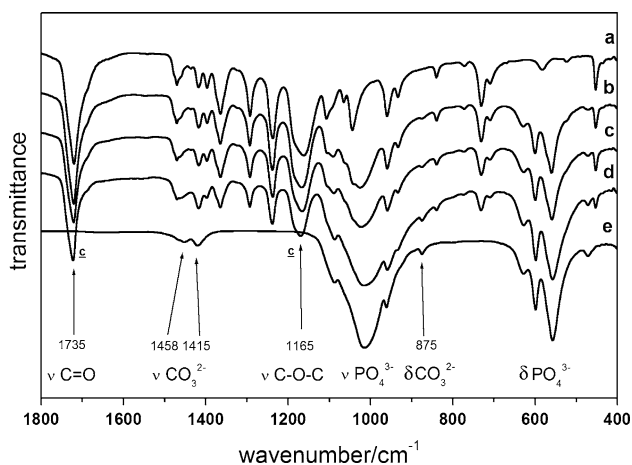


Fig. 2 IR spectra of the untreated samples: (a) PCL; (b) PCL/HA 70/30; (c) PCL/HA 60/40; (d) PCL/HA 50/50; (e) HA. Assignments: ν = stretching, δ = bending. The components mainly due to crystalline PCL are indicated by \underline{c}

the composites (Fig. 2e), are due to the CO₃²⁻ vibrations [22, 23, 29, 30] and indicate that the HA component was constituted by a B-type carbonated hydroxyapatite (i.e. carbonate ions substitute some phosphate ions in the lattice) (Fig. 1).

Raman spectra (Fig. 3) confirmed the IR findings, although the changes in the FWHM of the marker bands of PCL crystallinity were less evident. In particular, Raman analysis further confirmed that PCL/HA 50/50 had the most crystalline polymeric component (Fig. 3d). In this composite the components at 1735 cm⁻¹ (ν C=O) and 1090 cm⁻¹ (skeletal vibration), typical of amorphous PCL [31], were weaker than in the other samples; consequently, the narrowing of the bands at 1725 cm⁻¹ (ν C=O) and 1107 cm⁻¹ (skeletal vibration), sensitive to the PCL crystallinity, was observed, in agreement with the IR data. Moreover, the intensity ratio between the components of the doublet at 1285–1305 cm⁻¹ (I_{1285}/I_{1305}), due to the wagging vibration of the CH₂ groups (ω CH₂), increased (Fig. 3d). Since the component at lower wavenumber is attributable to crystalline PCL, while the other is due to

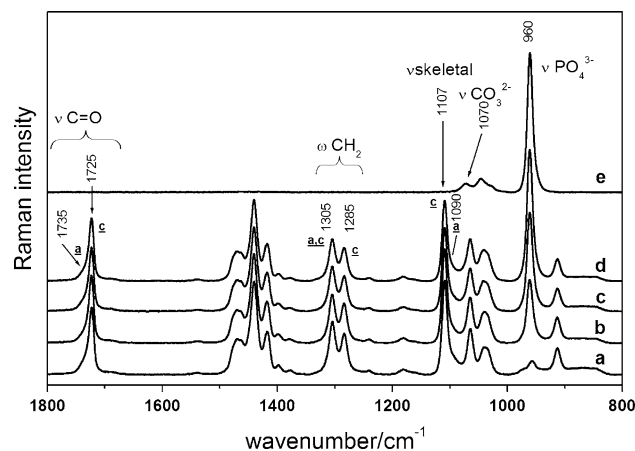


Fig. 3 Raman spectra of the untreated samples: (a) PCL; (b) PCL/HA 70/30; (c) PCL/HA 60/40; (d) PCL/HA 50/50; (e) HA. The components mainly due to crystalline and amorphous PCL are indicated by \underline{c} and \underline{a} , respectively. Assignments: ν = stretching, ω = wagging

both amorphous and crystalline PCL [31], this spectral change indicates a higher crystalline content in the PCL/HA 50/50 sample.

Typical Raman bands of HA were found in the 400–600 cm⁻¹ range (PO₄³⁻ bending modes), at 960 cm⁻¹ (very strong ν_1 PO₄³⁻ symmetric stretching vibration) and in the 1030–1070 cm⁻¹ region (Fig. 3), where the ν CO₃²⁻ stretching vibration at 1070 cm⁻¹ typical of B-type carbonated hydroxyapatite falls, besides PO₄³⁻ stretching modes [32–34]. According to the IR spectra, the inorganic bands (in particular ν_1 PO₄) progressively increased in intensity with respect to the PCL bands (i.e. the ν C=O band at 1725 cm⁻¹) at increasing HA contents (Fig. 3).

3.2 Irradiated samples

3.2.1 Thermal analysis

The thermal analysis of the composites irradiated up to 50 kGy showed that the irradiation treatment does not induce relevant changes in the polymer morphology;

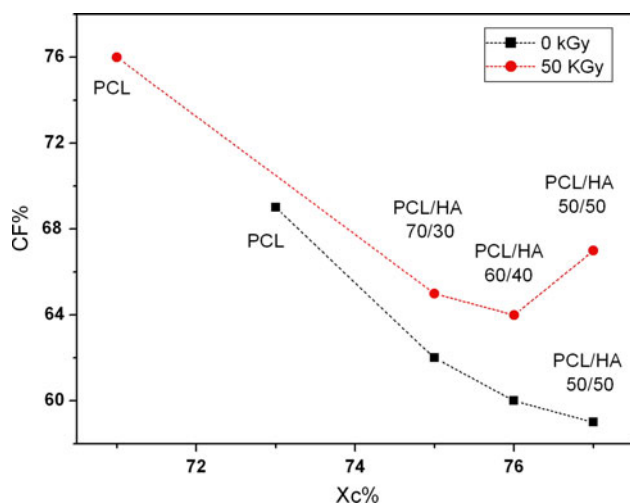


Fig. 4 Crystallisable fraction (CF%) versus crystallinity degree (X_c %) for PCL and PCL/HA samples before and after irradiation at 50 kGy in air

actually, the crystallinity of the polymeric component was poorly influenced by gamma-ray exposure in air, as indicated by the X_c values that remained quite constant after treatment (Fig. 4). On the contrary, the irradiation of pure PCL under the same conditions caused a decrease in crystallinity ($\sim 3\%$), suggesting a positive role of HA in preventing polymer modifications in the composites. This result can be explained in relation to previous studies which have demonstrated enhanced mechanical properties of the composites containing high amount of HA [35]. Analogous results were obtained after irradiation under nitrogen.

More interesting remarks can be made on the crystallisable fraction (CF%), which is in relation with the length of the polymeric chains. All the samples showed an increase in CF% (Fig. 4), PCL and PCL/HA 50/50 showing the most significant changes. This finding, along with a small decrease in the melting temperature (about 1°C) and a slight increase in the crystallisation temperature of the irradiated samples (in the 0.5 – 2°C range), suggests that the irradiation process caused a slight decrease in the polymer chain length. In fact, a polymer composed of shorter chains usually melts at a lower temperature and re-crystallises at a higher temperature, due to the increased molecular mobility.

The slight shortening of the polymeric chains by chain scission is probably due to the formation of electrons in the excited polymeric material upon gamma-ray exposure; the bond cleavage likely takes place at the level of the ester linkages, known as the sites most sensitive to irradiation in aliphatic polyesters [17, 36]. This result agrees with the literature: homopolymers, such as PCL, poly(lactic acid), etc., have been reported to degrade through chain-shortening at low doses of gamma-irradiation [17, 36]. On the

contrary, a random variation in PCL crystallinity and an increase in the crystallisation energy have been found to occur at doses exceeding 100 kGy, due to a higher extent of cross-linking [6, 15].

3.2.2 Vibrational analysis

Spectroscopic methods confirmed the occurrence of only very slight changes in the polymeric component of the samples, as evidenced by thermal analysis. The IR spectra of the irradiated samples did not show any significant change in FWHM of the marker bands of PCL crystallinity (i.e. the ν C=O and ν C–O–C bands) (Table 2). Moreover, the intensity ratio between the two bands at 1735 (ν C=O) and 559 cm^{-1} (δ PO_4^{3-}), taken as an index of the PCL/HA ratio, did not change after irradiation. As example, the IR spectra of the PCL/HA 50/50 scaffold, before and after 50 kGy gamma-irradiation under the two different atmospheres, are shown in Fig. 5.

A quantitative analysis of the PCL crystallinity was performed on the Raman spectra (see Fig. 6A as example). Attention was focused on the ω CH_2 doublet at 1285 – 1305 cm^{-1} , since the ratio between the areas of the two peaks can be taken as an index of polymer crystallinity [22, 23, 31]. The two bands were fitted in the 1250 – 1350 cm^{-1} range (Fig. 6A) and the ratios between their integrated areas were calculated for all samples and doses. The curve fitting analysis indicated that no significant variations occur in the scaffolds after gamma-irradiation (Fig. 6B).

With regards to the inorganic component, the ratio between the carbonate and phosphate ion content was investigated in all samples. The carbonate content plays a key role in determining the biological performance of the material since different types of calcified tissues (bone,

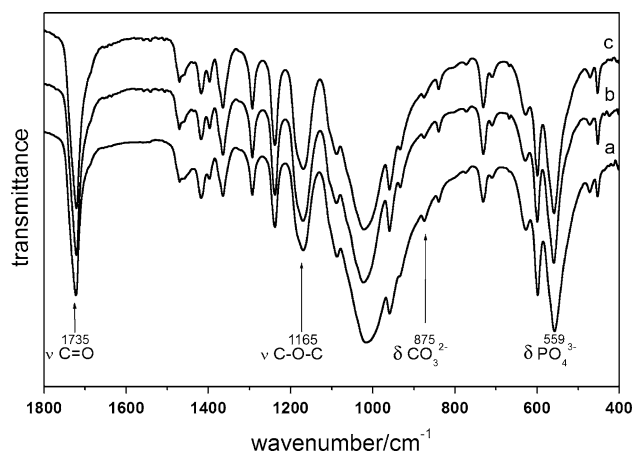
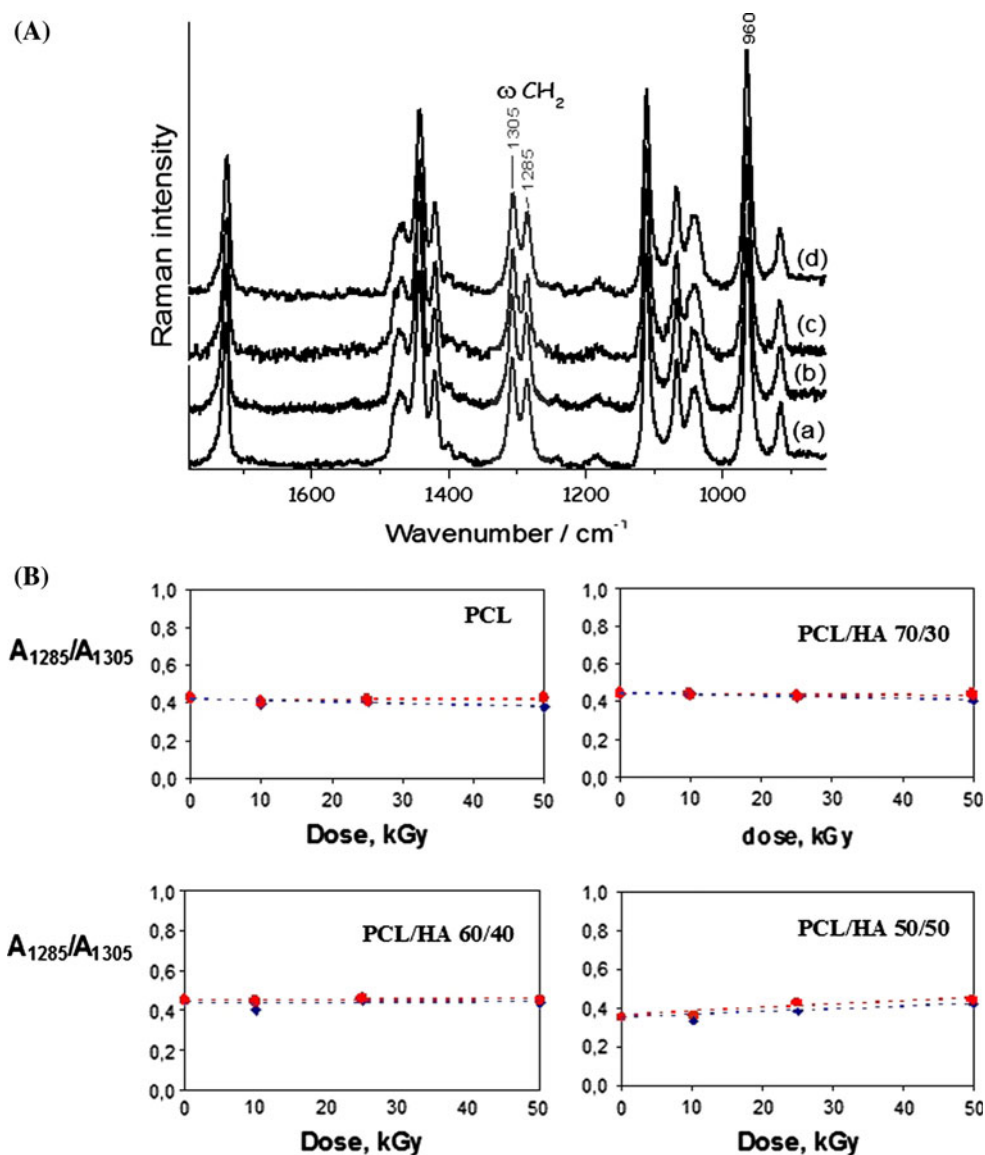


Fig. 5 IR spectra of PCL/HA 50/50 before (a) and after irradiation at 50 kGy under N_2 (b) and in air (c). Assignments: ν = stretching, δ = bending

Fig. 6 **A** Raman spectra of PCL/HA 50/50 (a) before and after irradiation in air at different doses: (b) 10, (c) 25 and (d) 50 kGy. **B** Trend of the ratio between the integrated areas of the bands at 1285 and 1305 cm^{-1} (A_{1285}/A_{1305}) versus irradiation dose in air (blue points) and under nitrogen atmosphere (red points). The integrated areas of the bands were calculated by curve fitting in the 1250–1350 cm^{-1} range and their ratio can be taken as an index of polymer crystallinity



dentin, enamel) show different carbonate concentration [37].

The $\text{CO}_3^{2-}/\text{PO}_4^{3-}$ ratio was calculated in the PCL/HA composites from the intensity ratio between the IR bands at 875 cm^{-1} (δCO_3^{2-}) and $\sim 559 \text{ cm}^{-1}$ (δPO_4^{3-}) (as an example, Fig. 5). The strongest carbonate bands of HA, visible at 1415 and 1458 cm^{-1} could not be used since in the PCL/HA spectra they are overlapped to the more intense bands due to the CH_2 deformation modes of the polymeric component (Fig. 2). The carbonate content resulted not to be significantly altered by gamma irradiation even at the highest dose.

Further confirmation was obtained by analysing the FWHM of the 960 cm^{-1} Raman band, sensitive to HA crystallinity [33, 34]: a perturbation of the crystalline organisation of HA determines a broadening of this band. The FWHM values resulted to change only a little without

a clear trend, thus confirming the conclusion obtained by IR data.

On the contrary, the high energy treatment on pure HA caused an increase in the amorphous content, as indicated by the broadening of the 960 cm^{-1} Raman band (Fig. 7A). This effect was confirmed by the IR spectra which showed an increase of the I_{875}/I_{559} ratio after HA irradiation in nitrogen and air (~ 22 and 25%, respectively) (Fig. 7B). This increase in the $\text{CO}_3^{2-}/\text{PO}_4^{3-}$ intensity ratio can be explained by considering that the crystallinity decrease of HA caused a decrease of the extinction coefficient of the phosphate bands.

Interestingly, the obtained results suggest that the polymeric component should protect the inorganic HA phase from the effects of gamma-irradiation.

It must be stressed that no significant differences were observed by irradiating the samples in air and under inert

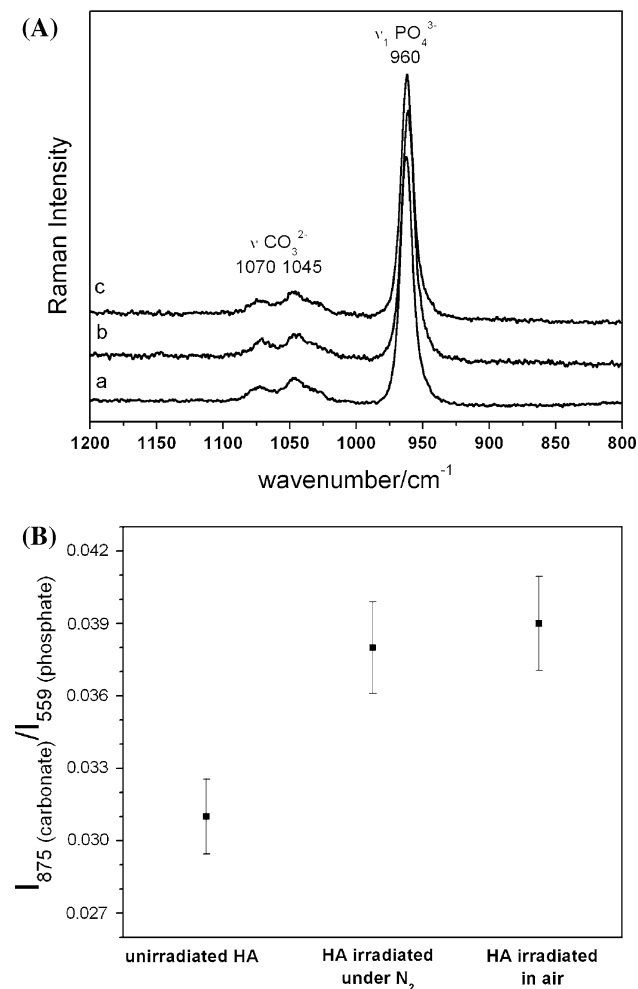


Fig. 7 **A** Raman spectra of pure HA before (*a*) and after irradiation at 50 kGy under nitrogen (*b*) and in air (*c*). Assignments: ν = stretching. **B** Intensity ratio between the IR bands of pure HA at 875 and 559 cm^{-1} (I_{875}/I_{559}) before and after irradiation treatment (50 kGy) in air and inert atmosphere

atmosphere, indicating that the radiation sensitivity of these scaffolds is independent of the presence of oxygen.

3.2.3 EPR spectroscopy

It is well known that radiations can induce paramagnetic defects in biological and synthetic carbonated hydroxyapatites [38, 39]. In this light, Electron Paramagnetic Resonance (EPR) measurements were carried out on irradiated PCL/HA scaffolds to obtain more information on the radiation-induced effects in the inorganic component of the composites.

All the treated samples, independently of the irradiation environment, exhibited similar anisotropic EPR spectra (Fig. 8). The untreated samples were analysed as control and proved EPR silent, indicating the absence of radical

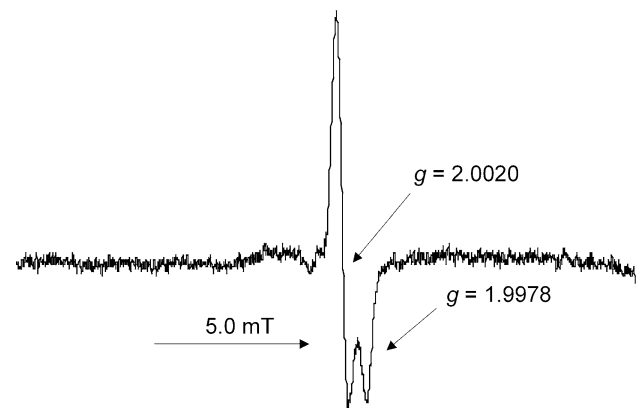


Fig. 8 Room temperature EPR spectrum exhibited by PCL/HA 50/50 after gamma irradiation (50 kGy) in air

species. As shown in Fig. 8, the EPR spectra of the irradiated samples consisted of two lines, the one at lower field ($g = 2.0020$) being much more intense than the one at higher field ($g = 1.9978$). This combination of signals is typical of the radical anion of carbon dioxide, $\text{CO}_2^{\bullet-}$ [40] and is exhibited by gamma-irradiated original bone tissue, either of animal or of human origin [39]; the former line is the component with perpendicular g (g_{\perp}) and the latter the component with parallel g (g_{\parallel}). The $\text{CO}_2^{\bullet-}$ radicals are due to the reaction between free electrons, produced by ionising radiation, and carbonated anions present in the biomaterial. These radicals are very stable with time and remain trapped in the hydroxyapatite matrix.

4 Conclusions

This work demonstrates that gamma-irradiation (up to 50 kGy administered dose) induces only minimal effects on the three-dimensional structure of different composite materials made of PCL and HA, used for bone substitution. In fact, both the polymer crystallinity and the relative composition of the samples were not significantly affected by the high-energy treatment.

The irradiation process caused chain scission in the composites which may slightly reduce the implant time of the material. This aspect can be positive since the PCL chains are bioresorbable; thus, the high-energy treatment may be a method for achieving a property enhancement in the PCL-based materials, as reported in other cases [41–43].

With regards to the mineral component, it was more sensitive to gamma irradiation than the polymer, because of the formation of stable paramagnetic radicals originating from the carbonate ions substituting PO_4^{3-} groups.

In addition, the atmosphere surrounding the composites during irradiation did not significantly affect the radiation

sensitivity of the PCL/HA scaffolds, since almost identical results were obtained in air or nitrogen.

In conclusion, the sterilisation process by gamma-ray irradiation did not significantly affect the investigated physico-chemical properties of the PCL/HA biomaterials, although it can be a step in the development and fabrication of tailored materials with shorter implant time. However, the choice of the synthesis parameters, in particular the content of carbonate anions in HA, has to be monitored to avoid the creation of too many defects in the biomaterial as a consequence of high-energy sterilisation treatment.

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