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Chemical-physical and preliminary biological properties of poly (2-hydroxyethylmethacrilate)/poly-(ε-caprolactone)/hydroxyapatite composite

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Abstract In the present study, the synthesis of a semi-Interpenetrating Polymer Network (semi-IPN) incorporating linear poly-(ε-caprolactone) (PCL) into cross-linked poly-(2-hydroxyethylmethacrilate) (PHEMA) reinforced with hydroxyapatite (HA) has been described. The aim of this study was to improve the mechanical and biological performance of the PHEMA/PCL in the hydrated state, for orthopaedic applications. The swelling behaviour, mechanical (compressive and tensile) and surface chemical-physical (morphology, stoichiometric composition) characterisation of the novel HA reinforced composite based on PHEMA/PCL polymer matrix, PHEMA/PCL 70/30 (w/w) + 50% (w/w) HA (PHEMA/PCL/HA), were evaluated. Furthermore, a preliminary *in vitro* biological evaluation was also performed on the composite using a fully characterised primary human osteoblast-like (HOB) cell model. The inclusion of HA in the composite improved the mechanical performance in the swollen state, with values of elastic modulus in a similar range to that of trabecular bone. The composite surfaces showed a porous, irregular topography with the presence of: oxygen (O), carbon (C); phosphorous (P); calcium (Ca) where the Ca/P ratio was 1.78. Biological evaluation indicated undetectable weight loss of the sample, no release of toxic leachables from the composite and pH values within an acceptable range for cell growth. The results indicate that the novel PHEMA/PCL/HA composite is a promising candidate as filler or substitute for spongy bone for orthopaedic applications.

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Introduction

Hydrogels are three-dimensional networks of hydrophilic polymers held together by covalent bonds and other cohesive forces such as hydrogen or ionic bonds. Glassy in the dry state, they swell extensively in water preserving their shape to form elastic gels. Their main property is the capability to retain a large quantity of water (from 20 to 95% of the total polymer weight) in their structure without dissolving. Other useful properties include: adjustable mechanical properties, the equilibrium water content, the possibility of tissue ingrowth into the polymeric matrix when implanted *in vivo*, thus making these materials very attractive candidates for biomedical use [1].

Poly-(2-hydroxyethylmethacrilate) (PHEMA) hydrogels have been successfully used since 1960 as biomaterials for medical applications in several fields, for example ophthalmology, plastic surgery, otolaryngology [1–3].

Both structural and chemical properties of PHEMA have been widely investigated, with significant results on both short- and long-term biological response to PHEMA hydrogels implantation. Generally it has been shown that PHEMA hydrogels are highly biocompatible but unfortunately, in the hydrated state, they are very soft with low mechanical strength and tear resistance, inadequate for biomedical applications where mechanical strength is a prerequisite [1].

The inclusion of semi-Interpenetrating Polymer Network (semi-IPN) of linear poly-(ε-caprolactone),

$[-O(CH_2)_5CO-]_n$, (PCL), a hydrophobic, non-toxic, semi-crystalline aliphatic polyester, into cross-linked PHEMA hydrogels has been shown to improve their mechanical properties in the swollen state, preserving at the same time, their softness, equilibrium water content, and permeability [1].

In order to achieve the specific mechanical properties required for orthopaedic application, a novel type of composite has been designed, reinforcing the PHEMA/PCL polymer matrix with hydroxyapatite (HA).

HA, or more specifically calcium HA, $Ca_{10}(PO_4)_6(OH)_2$, is a member of the compound family of apatite [4]: highly biocompatible, HA shows good bioaffinity, stimulates osteoconduction along its surface and is slowly replaced by host bone after implantation [4–6].

In the present study, a novel biologically acceptable composite for orthopaedic application PHEMA/PCL 70/30 (w/w) + 50% (w/w) HA (PHEMA/PCL/HA) has been developed and characterised. Swelling behaviour and mechanical performance of the material with tensile and compressive tests (dry and swollen conditions) were evaluated. Surface chemical-physical properties, in particular topography and stoichiometry composition, were also analysed.

Finally, a preliminary *in vitro* biological examination was performed analysing the loss of weight of composite samples following long term incubation in culture medium. In addition to pH analysis, cytotoxicity of the elutions was also evaluated.

Materials and methods

Materials

The composite (PHEMA/PCL/HA) was a semi-IPN composed of cross-linked poly-(2-hydroxyethylmethacrilate) (PHEMA) and linear poly-(ε-caprolactone) (PCL, average Mw: 65,000 Dalton; average Mn: 42,500 Dalton; Sigma-Aldrich) with monomer ratio of 70/30 (w/w) reinforced with 50/50 (w/w) ratio of HA (particle diameter: 2–10 μm, P205 grade, batch N1904, from IRC in Biomedical Material-QMW, London – U.K.).

The PHEMA/PCL blend was prepared by addition of PCL to 2-HEMA (Sigma-Aldrich) using a magnetic stirrer and controlled temperature conditions (water bath, $60 \pm 2\,^{\circ}C$).The homogeneous, transparent mixture was then gently mixed with HA at room temperature. Control samples were obtained without HA particles for the swelling behaviour characterisation.

The initiator α,α'-azobisisobutyrronitrile (AIBN) (purity $\geq$ 98% GC, Fluka), 0.05% (w/w), based on the weight of 2-HEMA, and the cross-linking agent ethylene dimethacrylate (EDMA) (Sigma-Aldrich), 0.01% (w/w) based on the weight of HEMA, were added and mixed. The resulting paste was poured into a rectangular silicon mould firmly sandwiched by two slides of polyester film and then by two slides of glass plates. In addition, cylindrical teflon moulds (5 mm diameter) were filled up with the same material. The moulds were cured at 90°C in the oven overnight (Mazzali, Vuototest). After cooling, the composite was washed with bidistilled H_2O (Carlo Erba) three times a day for 4 days, a blade was used to obtain: (a) flat-shaped surface discs (4 mm thickness; 12 mm diameter) from the rectangular sheet for swelling behaviour study, surface chemical-physical characterization and *in vitro* biological assays; (b) cylindrical samples (5 mm thickness; 10 mm diameter) from the cylindrical moulds for compressive characterisation; (c) dumbbell shaped specimens (length 22 mm, width 5 mm, thickness 1.5 mm) from the rectangular sheet for tensile characterisation. The samples were again washed as before for another 3 days, and finally dried in a laminar flow cabinet for 3 days.

Swelling behaviour

Cylindrical samples were dried overnight in a vacuum oven at 40°C and −1000 mbar (Mazzali, Vuototest). After cooling, at room temperature they were weighed and placed in distilled water at 23 ± 2°C. At selected time points, the samples were removed from water, and any excess water was removed by blotting on a tissue and the samples were weighed (Mettler Toledo AB54-S balance, 0.1 mg resolution). The water uptake has been calculated according the equation: $\Delta Wu = (w\text{-}w_0)/\ w_0$, where w and w_0 indicated the swollen and the dry weight of the specimen, respectively.

Mechanical characterisation

Tensile characterisation

Tensile ultimate strength (TUS), elastic modulus (TE) and maximum strain ($T\varepsilon_r$) were evaluated on microtensile dumbell geometry samples (five replicates) in dry (specimens were stored at 23°C) and swollen state (samples incubated in water, until the swelling equilibrium was reached). The speed of testing of 1.03 mm/min was used until the sample broke. The tests were carried out according to ASTM D1708 using an Instron testing machine model 4204 (load cell 1 kN; temperature of 23 ± 2°C; relative humidity 50 ± 5%). Elastic modulus was calculated both in dry and swollen state by a linear regression over a range of 0.005 mm/mm.

Compressive characterisation

Compressive ultimate strength (CUS), elastic modulus (CE) and maximum strain ($C\varepsilon_r$) evaluations were performed both

in swollen and in dry state on cylindrical samples (five replicates) according to ASTM D695 using an Instron testing machine model 4204 (load cell 5 kN; temperature 23 ± 2°C; relative humidity 50 ± 5%). The cross-head rate was fixed at 1.3 mm/min. Elastic modulus was calculated in the case of dry state by a linear regression over a range of 0.005 mm/mm, whereas a range from 0.8 mm/mm to the final value was chosen in the case of swollen state.

Surface chemical-physical characterisation

Surface topography

The samples were sputter coated with gold (Emitech 500, 4 minutes at 20 mA, 10^{-1} mBar) before examination under a Jeol JSM 5500 LV SEM at an accelerating voltage of 15 keV.

Surface chemistry

The samples were sputter coated (Emitech, K550) and then analysed by Energy Dispersive Spectroscopy (EDS) by using an Inca Energy 200 (Oxford Instruments, UK) for elemental analysis.

In vitro biological assays

Sample loss of weight

Samples previously dried and weighed were placed in a 24 well plate for tissue culture. 1 ml of culture medium [Dulbecco's Modified Eagles Medium (DMEM) (Sigma) containing: 10% foetal calf serum (FCS) (Sigma), 1% non-essential amino acids (Sigma), 150 μg/ml ascorbic acid (Sigma), 2 mM L-glutamine (Sigma), 100 units/ml penicillin (Sigma), 0.100 mg/ml streptomycin (Sigma), 0.01 M (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (Sigma)] was then added. The plates were incubated at 37°C with 5% CO_2 in a humidified atmosphere. At selected time points, the specimens were removed from the culture medium, excess water was wiped off with a tissue, dried overnight at 37°C and then weighed (Mettler Toledo AB54-S balance, 0.1 mg resolution).

The weight loss measurement has been expressed as $\Delta W_L\% = [(w_2\text{-}w_1)/ w_1]^*100$, with w_1 and w_2 indicating the weight of the dried specimen before and after the incubation, respectively.

Elution measures

The samples (four replicates) were placed in 10 ml universals containing 5 ml of culture medium and incubated at 37°C on a rotating mixer. At the selected time points the aqueous extracts were collected, replaced with 5 ml of fresh medium and the universals placed back at 37°C. The pH of the eluted media (in triplicate) was measured using a pH meter (Corning 240).

Elution cytotoxicity assay

Primary human osteoblastic-like cells (HOBs) were isolated from trabecular bone using a previously described method [7]. Briefly, the bone chips were cultured under sterile conditions until "osteoid seams" were seen then digested using collagenase (100 U/ml) (Sigma) and trypsin (300 U/ml) (Sigma) solution. Following isolation, expansion and characterisation, the obtained cells were used for the cytotoxicity tests. The samples (four replicates) were placed into a 24 well tissue culture plate, flooded with 1 ml of culture medium and incubated at 37°C with 5% CO_2 in a humidified atmosphere. At the selected time points the aqueous extracts were collected, replaced with 1 ml of fresh medium and the plates placed back in the incubator. 100 μl of 1×10^5 cell/ml cell suspension were seeded into a sterile 96 well culture plate (Benton Dickinson) and incubated to confluence. The culture medium was then replaced with aqueous extract (100 μl /well; three replicates) and the plates were incubated at 37°C with 5% CO_2 in a humidified atmosphere for 48 and 72 hours. 10 μl of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2 H-tetrazolium bromide (MTT) (Sigma) solution (5 mg/ml in phenol red- and ascorbic acid-free culture medium containing FCS) was added to each well, the plates were incubated for 4 hours and then the medium removed by inversion. 100 μl of DMSO (Merck) was added to each well, mixed for 20 minutes until complete dissolution of the crystals was achieved. Absorbance was measured by a Dynatech MR 700 plate reader (test wavelength 570 nm; reference wavelength 630 nm).

Results

Swelling behaviour

The composite samples showed an increasing trend with time. The equilibrium condition was reached after 44 hours corresponding at a gain of 20,3% in weight (0.203 ± 0.002) while samples without HA showed an equilibrium water uptake of about 28% (0.028 ± 0.003) (Fig. 1).

Mechanical characterisation

Tensile characterisation

Tensile tests in dry state showed a brittle mechanical behaviour with an ultimate strength of 14.32 MPa and a maxi-

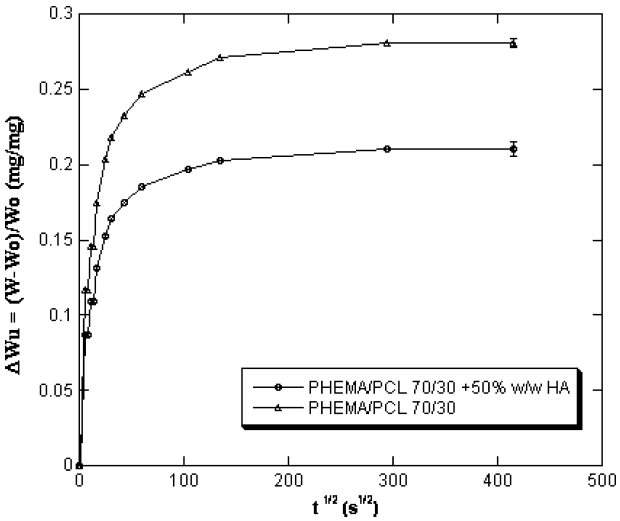

Fig. 1 Water uptake of the PHEMA/PCL 70/30 (w/w) sample and PHEMA/PCL 70/30 (w/w)+50% (w/w) HA composite in term of $((W-W_0)/W_0)$ as function of the square root of the time (s 1/2)

mum deformation of about 0.7%; also an elastic modulus of 2027.51 MPa was calculated (Fig. 2a).

In swollen state, an increase of maximum deformation (from about 0.7 to 19%) and also a reduction of elastic modulus (from 2027.51 to 19.92 MPa) (Fig. 2b) were observed.

The results are summarised in the Table 1.

Compressive characterisation

Compressive tests in dry condition showed a brittle mechanical behaviour with an ultimate strength of 48.05 MPa and a deformation at maximum strength of 0,1204 mm/mm; an elastic modulus of 909.15 MPa was also calculated (Fig. 2c).

Compressive stress-strain curve in wet condition showed two regions: from 0 to 0,75 mm/mm, a very slow increase of slope was observed followed by a sharp increase of stress values up to 0.9 mm/mm (Fig. 2d).

The results are summarised in the Table 1.

Surface chemical-physical characterisation

Surface topography

The PHEMA/PCL/HA surface (Fig. 3) was variable; rough, porous, with grainy particles ranging in size between 2 and 10 μm and several very smooth areas. The morphology was not uniform with both flat and very grainy regions.

Surface chemistry

The EDS analysis of the composite sample surfaces showed the following elements: oxygen (O), carbon (C); phosphorous (P); calcium (Ca). The Ca/P atomic ratio values obtained for the composite was 1.78, close to both the typical value of stoichiometric HA and the value obtained for the HA used for the test composite preparation [8] (Fig. 4).

In vitro biological assays

Sample loss of weight analysis

The composite samples stored in culture medium showed undetectable decrease in weight up to 42 days of incubation.

Elution pH determination

No significant differences in the pH values of the eluates was observed, remaining approximately in the range 8–9 from day 1 to day 42 of sample incubation in culture medium.

Elution cytotoxicity assay

The comparison between the controls, TMX, non-toxic control, PVC, toxic control and the eluates collected from the composite samples (Fig. 5) at the selected time points showed no evidence of cytotoxicity after 48 and 72 hours of incubation with HOB cells.

Discussion

Swelling ability in different solvents depends on the chemical structure, presence of ionic groups, type and density of cross-links. The degree of hydrogel swelling depends on several factors, favourable (osmotic potential; strong interaction with water; high free volume; high chain flexibility; low cross-link density) and inhibitive (weak interaction with water; low free volume; low chain flexibility; high cross-link density).

In particular, equilibrium swelling analysis reported in literature indicates that, in PHEMA/PCL semi-IPN, the PHEMA component retains its high water content characteristic, positive attribute that makes PHEMA hydrogels highly biocompatible [1].

In the present study, the swelling behaviour analysis performed on PHEMA/PCL/HA composite revealed an increasing trend up to 44 hours with a 20.3% of equilibrium percent water absorbed. This is remarkably lower than the 40% reported for pure PHEMA hydrogels [9]; the decrease of ΔWu can be the result of the PCL component in conjunction with the presence of the HA in the composite.

In fact, the equilibrium water uptake decreases with increasing weight percent of PCL in PHEMA/PCL semi-interpenetrating polymer networks compared to PHEMA

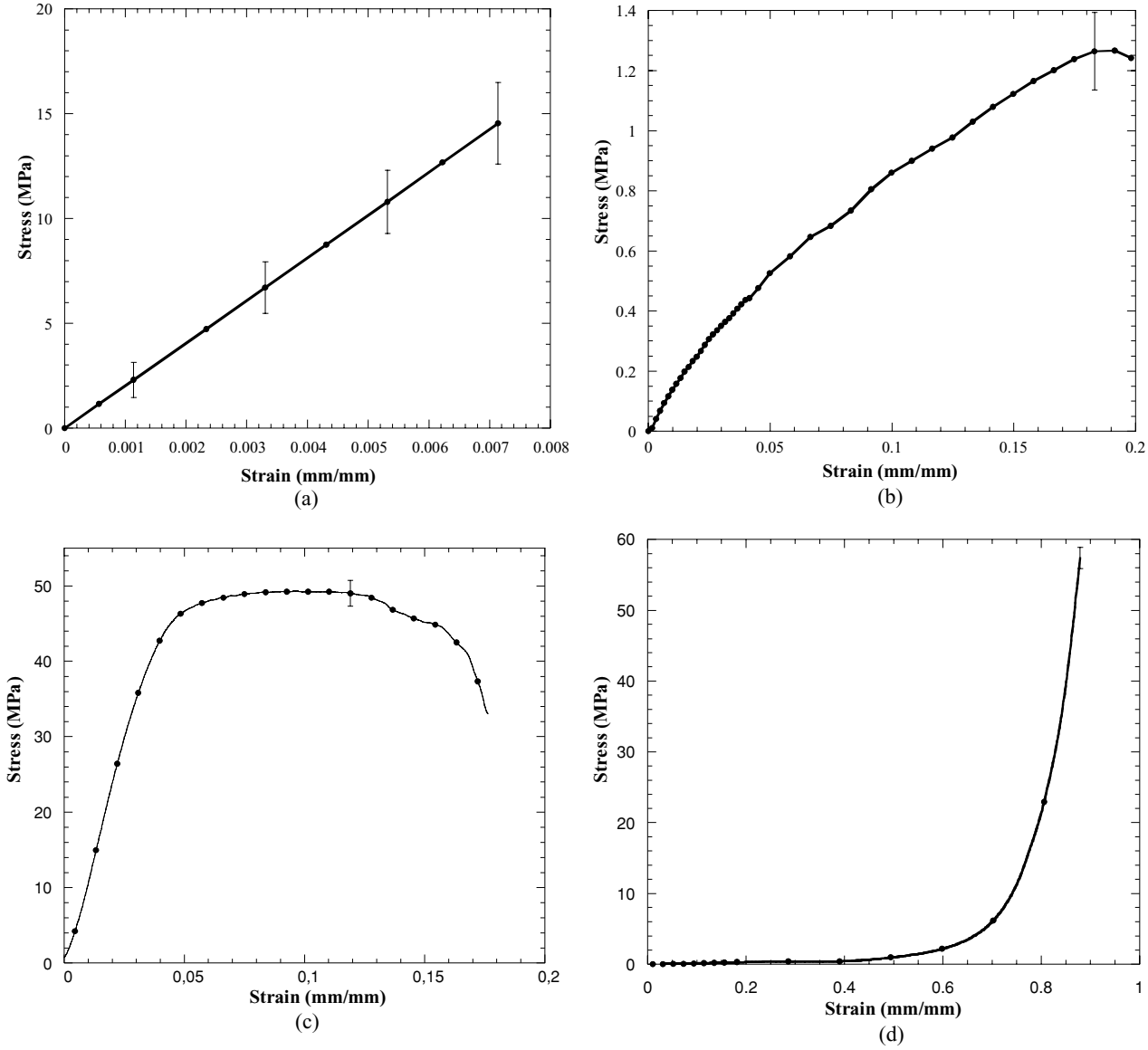

Fig. 2 Stress-strain curve of the PHEMA/PCL 70/30 (w/w)+50% (w/w) HA composite (a) tensile test, dry state (b) tensile test, swollen state (c) compressive test, dry state (d) compressive test, swollen state

hydrogels [1]. In particular, the value achieved in the present work for PHEMA/PCL samples was 28.08%.

The further reduction in terms of equilibrium percent water absorbed from 28.08 to 20.3% can be ascribed to the of HA particles as well as probably to the presence of voids in the composite structure (Fig. 1).

In addition, the increase in the hydrophobic properties provides a stiffer polymer backbone resulting in the reduction of chain extension, which in turn decreases the net swelling of the composite.

Mechanical properties play a key role in natural bone tissue that needs to resist the loads placed on the skeleton. Compressive, tensile and shear forces have been estimated on bone; in particular, trabecular bone functions mostly in compression whiles compact bone functions in compression, tension and shear [10–11]. As a consequence, in order to restore the injured skeleton mechanical integrity, the mechanical performance of an implanted material should be comparable to the replaced type of bone. In particular it has to be guaranteed a suitable mechanical performance in water-swollen state, being the working condition upon implantation in the human body [12–13]. For these reasons, the mechanical properties have to be assessed not only in dry but also in wet condition.

Tensile test performed on dry PHEMA/PCL/HA composite showed a brittle mechanical behaviour with an ultimate strength of 14.32 MPa, a maximum deformation of about 0.7% and an elastic modulus of 2027.51 MPa. A remarkable

Table 1 Mechanical properties of the PHEMA/PCL 70/30 (w/w)+50% (w/w) HA

Tensile	Dry state	Tensile Ultimate Strenght TUS (MPa)	14.32 ± 1.95
		Tensile Elastic Modulus TE (MPa)	2027.51 ± 113.42
		Tensile Maximum Strain tε_m (mm/mm)	0.0073 ± 0.0008
	Swollen state	Tensile Ultimate Strenght TUS (MPa)	1.26 ± 0.18
		Tensile Elastic Modulus TE (MPa)	19.92 ± 1.05
		Tensile Maximum Strain tε_m (mm/mm)	0.1915 ± 0.093
Compressive	Swollen state	Compressive Ultimate Strenght CUS (MPa)	57.36 ± 1.53
		Compressive Elastic Modulus CE (MPa)	440.76 ± 0.92
		Compressive Maximum Strain cε_m (mm/mm)	0.8789 ± 0.0540
	Dry state	Compressive Ultimate Strenght CUS (MPa)	48.05 ± 1.71
		Compressive Elastic Modulus CE (MPa)	909.15 ± 84.25
		Compressive Maximum Strain cε_m (mm/mm)	0.1204 ± 0.0132

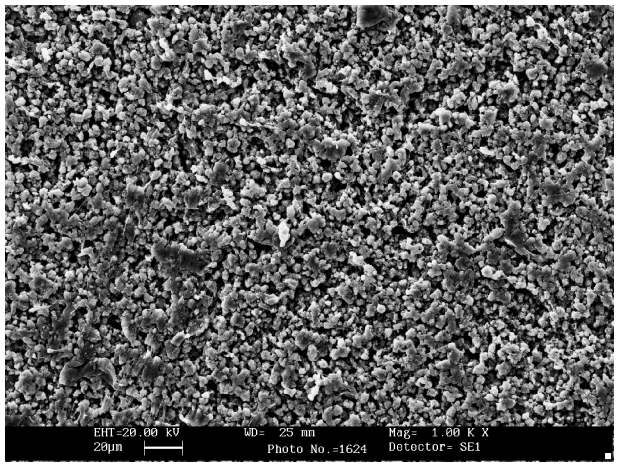

Fig. 3 SEM micrographs of the PHEMA/PCL 70/30 (w/w)+50% (w/w) HA composite sample surface

difference in the composite mechanical properties was observed when tested in water-swollen state, resulting in an increase of maximum deformation (from about 0.7 to 19%) and a reduction of elastic modulus (from 2027.51 to 19.92 MPa). Compressive stress-strain curve in swollen state showed, from 0 to 0.75 mm/mm, a very slow increase of the stress-strain values, *toe region,* due to the hydrophilic matrix of the composite constituents under an increasing compressive deformation. As the strain exceeds the threshold of 0.75 mm/mm, a rapid increase of the stress value was reported up to 0.9 mm/mm, indicating a stiffening of the structure (elastic modulus 440.76 MPa) with a maximum strain of about 0.87 mm/mm. A more brittle behaviour was achieved in dry state with an increase of elastic modulus from 440.75 to 909.15 MPa and a decrease of maximum strain (from 0.87 to 0.12 mm/mm).

These results agree with the data reported in literature for PHEMA/PCL semi-IPN, that also underlines the dependence of the described effect, on the amount of PCL present in the semi-IPN composition [1]. The presence of HA provides higher mechanical properties for the PHEMA/PCL semi-IPN structure. Indeed, HA increases the stiffness by rigid particulate dispersion inside the composite structure. This phenomenon is strongly dependant on the amount of HA and remarkable in the studied case of 50%(w/w) HA percentage.

These results indicate that PHEMA/PCL/HA has mechanical properties in the range of that shown by that of spongy bone [14]. Hence, the results from the mechanical characterization of PHEMA/PCL/HA composite makes it a suitable candidate to spongy bone augmentation or substitute.

Surface topography is the first parameter that determines cellular response on contact with biomaterial and subsequently its integration and long-term performance once implanted *in vivo*. Topography affects important parameters involved in the cell-material interaction such as: protein and cellular adhesion; cell movement, orientation, morphology; cellular cytoskeleton; contact inhibition; phagocytosis; gene expression [15–16]. It has been reported that, *in vivo*, the bonding strength between bone and implant materials is greater for rough composite materials because they provide a surface that favours cell adhesion [17]. Also, a phenomenon known as rugophilia has been described, whereby a roughened surface increases cell, and, in particular, osteoblast response to materials [17–18]. PHEMA/PCL/HA sample surface was not uniform, showing irregular flat and very grainy areas, most probably the result of the sandwiching needed for the composite specimen preparation. This could be an advantage and might provide a suitable surface topography for bone cells adhesion and proliferation. Surface stoichiometry is an important requirement for biomaterials to attract the ECM constituents involved in bone cell adhesion [19–20]. In particular, it has been proposed that by exposing HA, well known to be osteoconductive, on the material surface directly in contact with cells might create a suitable environment for osteoblast adhesion [21–25].

Surface stoichiometry analysis on the test materials indicated the presence of expected atomic component of the composites: O, Ca, P, C.

The Ca/P ratio obtained indicated the presence of HA directly exposed on the surface of the samples, suggesting that

Element	Weight %	Atomic %
C K	27.61	41.42
O K	36.36	40.94
P K	10.89	6.33
Ca K	25.14	11.30

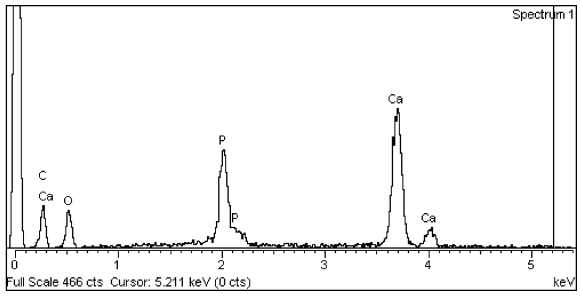

Fig. 4 EDS analysis on of the PHEMA/PCL 70/30 (w/w)+50% (w/w) HA composite sample surface

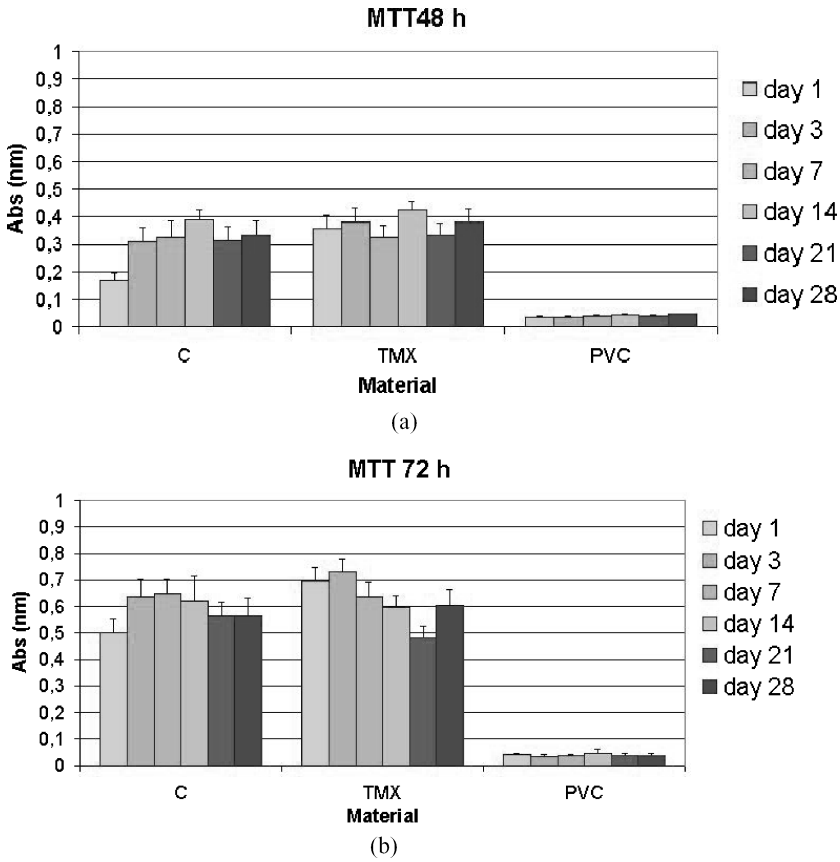

Fig. 5 MTT test after (a) 48 hours and (b) 72 hours of HOB cell incubation with composite samples (c) eluted in culture medium for 1; 3; 7; 14; 21; 28 days

this topographical HA presentation might allow the cells to react positively to the composite surface microenvironment. It has to be underlined that the values obtained from the EDS analysis were semi-quantitative because the surface of the "as cast" samples was irregular and no specific treatments were used to smooth the surface. The EDS analysis was performed directly on the "as cast" surface in order to obtain informations relative to the "real" situation presented to the cells once seeded *in vitro*.

Cellular responses are known to be affected by the modification of material surface properties and also the release of leached products due to degradation process that occurs on incubation in an aqueous biological aggressive environment such as human body [1; 26–27]. Furthermore, it has been documented in the literature that pH changes of the surrounding environment affect cellular activity: at alkaline pH (8,5–9) the cells are "stimulated" but they are affected if pH is above 9 [28].

The pH measurements of the test material supernatants for all the selected time points ranged from approximately 8 to 9. These results indicated that the reactivity of the material with the surrounding fluids did not create an unfavourable microenvironment for HOB cells activity.

These results showed that there was no detectable composite degradation and no release of toxic leachables from PHEMA/PCL/HA composite eluted over the selected periods of time. Surface analyses showed an irregular topography with very smooth and porous, grainy areas. The Ca/P ratio indicated the presence of HA exposed on the composite surface, which provided a suitable environment for cell adhesion.

The swelling behaviour of PHEMA/PCL/HA composite showed a 20,3% of equilibrium percent water absorbed revealing an increasing trend up to approximately 2 days.

The *in vitro* elution cytotoxicity evaluation showed no evidence of sample degradation or toxic leachables from the composites and acceptable pH values for the eluted media.

Conclusions

The present study has shown that the mechanical properties of PHEMA/PCL/HA are comparable to that of spongy bone. HA increased the stiffness by rigid particulate dispersion inside the composite structure, acting as the ceramic component of bone. Furthermore, the local pH environment did not have a detrimental effect on cell activity and no cytotoxicity was observed. Hence, the results indicate that the novel PHEMA/PCL/HA composite could play an interesting role as novel material for orthopaedic applications.

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