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Bioactive Agrocomposite for Tissue Engineering and Bone Regeneration

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Abstract: Background: This study describes a novel biomaterial consisting of a mixture of biphasic bioceramic obtained from waste generated by the sugar industry (Carbocal) and a medical-grade epoxy resin adhesive called LOCTITE® M31 CLTM. The objective was to demonstrate the possibility of coating non-bioactive and non-biodegradable metallic surfaces on implantable elements. Methods: After preparation, the mixture was applied to the surfaces of hip prostheses composed of two distinct materials: polyetherimide and grade 5 titanium. In both cases, adhesion tests produced favourable results. Additionally, cell cultures were conducted using human foetal osteoblastic cell lines (hFOB 1.19). Results: It was observed that the mixture did not affect the proliferation of bone cells. Conclusions: This composite material was found to promote the growth of bone cells, suggesting its potential for fostering bone tissue development.

Keywords: biphasic bioceramic adhesive; Carbocal; epoxy resin; cell viability; bone tissue engineering; biocompatible coating

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1. Introduction

Bone tissue engineering is a multidisciplinary field that combines regenerative medicine, biomechanics, and materials engineering [1–3]. The complexity of bone tissues and their limited capacity for natural regeneration make bone tissue engineering a crucial area of research in medicine [1–3].

Regeneration and the repair of bone defects are central goals in this evolving field [4,5]. Scaffolds, used for bone repair or replacement, play a crucial role in this biomedical discipline [6–8]. In this context, the selection of materials for scaffolds represents a significant challenge [9–11]. The introduction of biomaterials in bone tissue repair has revolutionised therapeutic approaches [12–14]. These materials not only act as structural supports but can also modulate cellular and tissue response to promote bone regeneration [12–14].

In this context, hydroxyapatite (HA)-based scaffolds have shown promise for bone regeneration [15–20]. HA, being a natural component of bone tissues, it exhibits excellent biocompatibility [21–23]. However, improving its mechanical and adhesion properties remains a major challenge in bone tissue engineering [15–20].

Obtaining HA from traditional natural sources presents ethical and environmental concerns [24]. Therefore, the exploration of new sources for HA synthesis has become

crucial. One promising approach is the use of waste from the sugar industry, in line with circular economy and sustainability principles [25–27].

A recent study has described the production of a biphasic bioceramic from Carbocal®, a by-product of the sugar industry [27]. This bioceramic shows similar properties to commercial HA, suggesting its potential for biomedical applications. In addition, it is composed of 24% TCP (tricalcium phosphate, $Ca_3(PO_4)_2$), known for its osteoconductivity and biodegradability. Using Carbocal® instead of animal bones for the production of bioceramics can reduce waste and promote the circular bioeconomy [27].

The processing of sugar beet to produce sugar generates three types of by-products: sugar beet leaves, dry pulp, and Carbocal®. These by-products are used as fertilisers, animal feed, and even in the production of cement for the construction sector. However, the production of these by-products is very high, reaching 80% of the raw material, amounting to 250,000 tonnes per year, so a large fraction of them usually go to landfills or become incinerated, resulting in a consequent imbalance of CO2 production. This is why the availability of this by-product would not generate a bottleneck in its application. Specifically, Carbocal®, due to its $CaCO_3$ content and its physico-chemical characteristics, is very interesting as a hydroxyapatite precursor. It is a product consisting of more than 80% $CaCO_3$, 7% organic matter, oligo-elements, and assimilable organic acids.

In addition to advances in materials, surface modification and coating techniques for metallic implants are fundamental in bone tissue engineering [28–35]. However, some of these techniques have limitations, such as weak bonding between implants and coatings [28–35].

The development of adhesive hybrid biomaterials and biocomposites offers a promising alternative [28–35]. These materials can avoid problems associated with metal-based implants, such as corrosion and the need for subsequent removal [28–35].

Additionally, a comparison of the bonding performance of some commercial adhesives for medical use in dental applications was established [36], where it was concluded that the adhesive called LOCTITE M31CL had the best performance, in terms of bond strength on materials used in additive manufacturing (3D printing). Since the 1960s, acrylic cements and those based on polymethylmethacrylate (PMMA) mixtures that polymerise quickly and are applied directly on the implant itself have been used. Unfortunately, bone removal processes are common in areas where prostheses made from these materials were previously implanted, mainly due to corrosion phenomena. The biomaterials and adhesive hybrid biocomposites resulting from the processing described in this work avoid the main problems that arise with implants based on metallic materials. Materials such as stainless steel, titanium alloys or chromium–cobalt–nickel alloys do not guarantee bone formation and cause problems in the vicinity of surgery and, and most importantly, give rise to corrosion phenomena. The work presented here demonstrates the possibility of coating non-bioactive and non-biodegradable metallic surfaces on implantable elements with elements of these specific characteristics, which have the additional guarantee of not suffering detachment or cracks due to corrosion.

This study describes the synthesis of a biomaterial consisting of a mixture of biphasic bioceramic and an epoxy-resin-based adhesive for the priming of implantable surfaces [28–35]. This biomaterial could have bioactive potential, inducing cell nucleation and growth on the implant surface, regardless of its metallic or non-metallic composition.

The literature consulted does not report cases of obtaining a biomaterial for the priming of metallic or non-metallic implantable surfaces, consisting of a mixture of BBC and an epoxy-resin-based adhesive, such as the one described in this work, except the patent applied for by the authors [37]. This biomaterial could potentially play a bioactive role, having the ability to induce cell nucleation and growth on the biomaterial surface, regardless of whether it is metal or non-metal.

2. Materials and Methods

For this study, Carbocal® waste was acquired from the sugar industry in the province of Cadiz (Andalusia, Spain) in 2021. Carbocal® was obtained from AB Azucarera Iberia S.L. (Jerez de la Frontera, Cádiz) directly, as it results from sugar refining, which is a suitable by-product due to its natural characteristics, annual production volume, and consideration as a waste, without guaranteed valorisation. Table 1 summarises its chemical properties and the generation process.

Table 1. Characteristics of Carbocal® (CB) [38].

Morphology	**Powder**
Genesis	Purification process of juice sweetened with lime hydroxide and CO_2
Chemical composition	>80% $CaCO_3$; 7% organic matter; oligo-elements (N, K_2O, P_2O_5 and Mg); assimilable organic acids
Humidity	<35%
Volume	20,000 average annual tonnes

The adhesive used is a medical grade epoxy called LOCTITE® M31 from the manufacturer HENKEL IBÉRICA, S.A. (Alcalá de Guadaíra, Seville, Spain), and it was selected from 5 products, based on the results obtained from the adhesion test previously carried out in another study [39]. It is a two-component adhesive based on a resin (R) and a hardener (H) (ratio R/H = 2/1), whose mixture is almost transparent in colour; it has low viscosity and a specific gravity of 1.07 kg/cm^3 at 25 °C.

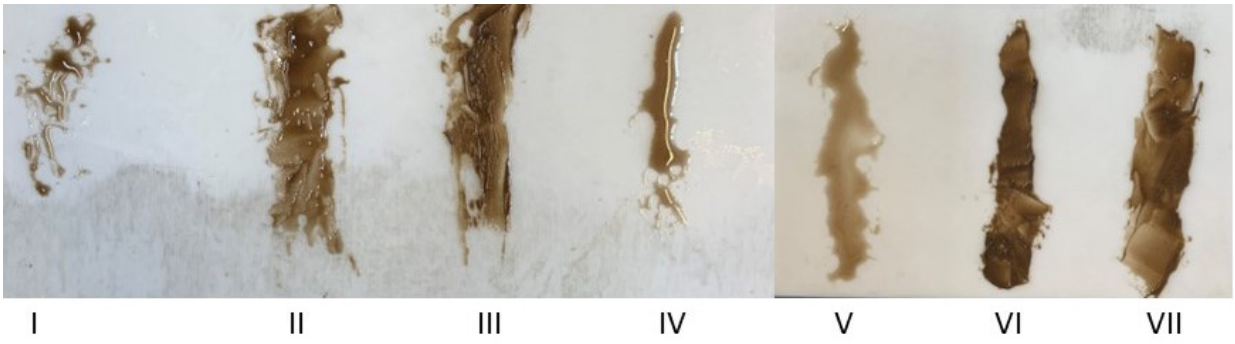
The BBC powder was obtained by hydrothermal phosphatization of Carbocal® [16,34], using NaH_2PO_4 as potassium source and respecting the molar ratio Ca/P = 5/3. The complete process of obtaining BBC is described in a previous publication [27].

Next, the BBC was mixed manually with the biocompatible epoxy adhesive until a greenish-brown biomaterial with a homogeneous texture was obtained. The mixing with the adhesive was carried out in different proportions in order to study the influence of this parameter on the behaviour of the resulting material. The prepared ratios are shown in Table 2.

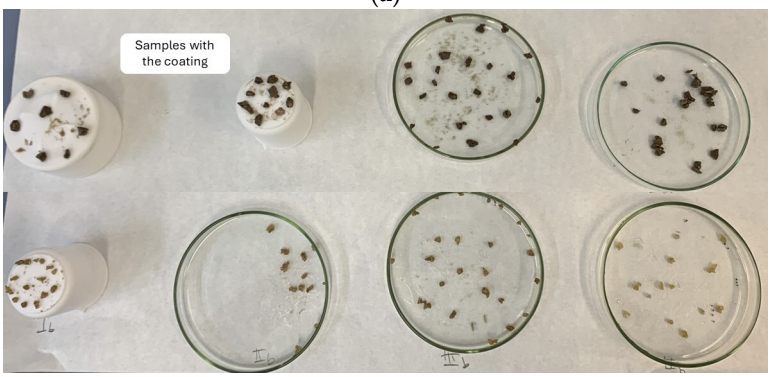
Table 2. Mass (milligrams) of each component and % composition of the different mixtures pre-pared.

Components	**Mix (mg)**							
	I	**II**	**III**	**IV**	**V**	**VI**		**VII**
H	135.5	133.5	133	138.2	275	345.3	H	310
R	276.7	275.4	264	266.1	566.1	720	R	740
BBC	100	199.7	300.4	40.9	843.6	1300.5	BBC-SBP	871
Total	512.2	608.6	697.4	445.2	1684.7	2365.8	Total	1921
% Composition								
BBC (%)	19.52	32.81	43.07	9.19	50.07	54.97	BBC-SBP	45.34
Epoxy (%)	80.48	67.19	56.93	90.81	49.93	45.03	Epoxy	54.66

In addition, a sample (VII) was prepared with BBC powder previously combined with SBP (Sugar Beet Pulp) and then mixed with epoxy in the proportion indicated in Table 2. These mixtures were cured for 90 min at 30 °C and withstood the autoclave sterilisation process in up to 80 °C without deformation or phase changes (Figure 1a).



(**a**)



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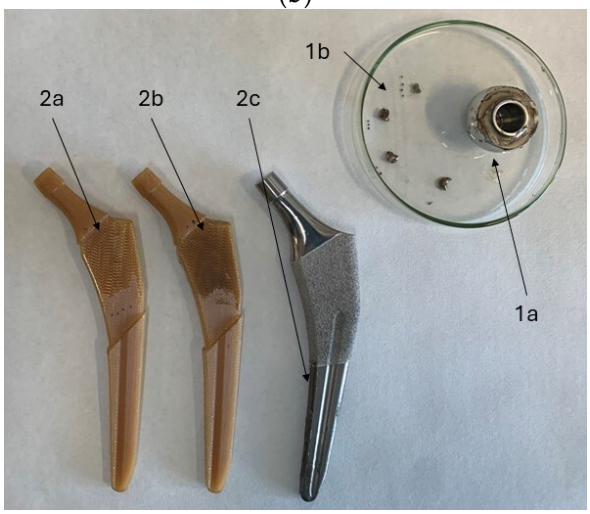


(**c**)

Figure 1. Sample preparation: (**a**) composite materials formed before coating the samples. Nomenclature I–VII corresponds to the compositions indicated in Table 2; (**b**) samples after coating 3 implantable surfaces (PEI-ULTEM1010® from the manufacturer Sabic (Riyadh, Saudi Arabia), Vitalium® from the manufacturer Dentsply Sirona (Charlotte, NC, USA), and Ti-6Al-4V); (**c**) implantable surfaces with adhesion test coatings: 1a—on femoral head on Vitalium material; 1b—different concentrations on small samples of Vitalium; 2a—epoxy only on flat surface of the intermediate part of the femoral stem of a hip prosthesis, manufactured in fused deposition (FDM) in material U1010; 2b—mixture VII on flat surface of the intermediate part of the femoral stem of a hip prosthesis, manufactured in FDM in material U1010; 2c—mixture VII on flat surface of the distal part of the femoral stem of a hip prosthesis, on loan from Stryker Iberia S.L. (Alcobendas, Madrid, Spain) in Ti-6Al-4V material.

To test the adhesion, on the implantable surfaces, 3 types of hip prostheses surfaces were used. Figure 1c shows a femoral stem piece made in Ti-6Al-4V (Ti5) and a femoral head made in Vitalium material (Co: 60,6; Cr: 31,5; Mo: 6,0; rest: Si, Mn, C), in accor-

dance with commercial Accolade II prosthesis by Stryker® Orthopedics (Stryker Iberia, Alcobendas, Spain) [40]. The third implantable surface used to study the coating is a commercial polyetherimide (PEI ULTEM1010®, U1010 in abbreviated form) with thermoplastic behaviour, with the following molecular formula: $[C_{37}H_{24}O_6N_2]$; molecular weight—592.61 g/g-mol; and density—1275 kg/m^3. U1010 was supplied by Sabic and optimised for a Fortus 450 MC machine by Stratasys (Eden Prairie, MN, USA) in the XY orientation (flat), as described in references [40–42]. Once the coatings were applied with a brush, both prostheses were kept in an oven at 37 °C for 12 h.

Grid-Cut Testing [43] was performed to ensure adhesion of the coating to the biomaterial, one based on the UNE-EN ISO 2409:2021 standard [44]. For this, once the coating was applied on a series of prosthesis surfaces, 6 perpendicular incisions were made between them, 3 to 3. The incisions were made with a V-shaped blade at 30°, spaced evenly at 3 mm. Figure 2 shows the instrument fitted with the blades for carrying out the test. This test was applied to the comparative materials studied (PEI, Ti-6Al-4V "Ti 5", Vitalium®) to select the most stable adhesion. After the test, the results are classified with Table 1 of the UNE-EN ISO 2409 standard based on grades 0 to 5, with 0 being optimal.

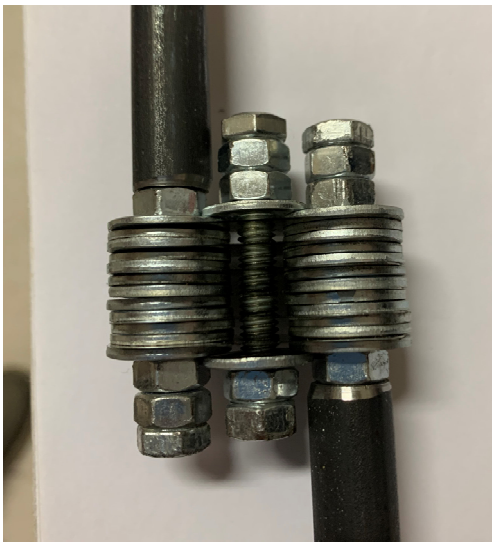


Figure 2. Instrument with V-shaped blades at 30° used for the grating in the adhesion test.

Cell cultures were performed with human foetal osteoblastic cell lines (hFOB 1.19), in the presence of the generated biomaterials, to determine whether the materials affected cell viability. For this cell viability study, hFOB cells were cultured in osteogenic media, and viability assays were performed at 24 h, 48 h, 72 h, and 7 days. In this assay, cells were incubated with MTS, and absorbance was subsequently quantified. Before the process, as a negative control, hFOB cultures were incubated with 70% methanol for 30 min. As a positive control, hFOB cultures without any treatment were used. The bio-compatibility of the materials was tested with primary human hFOB osteoblastic cell cultures. Normal human foetal osteoblasts were acquired from ATTC (hFOB cell line 1.19, CRL-11372TM). The hFOB cultures were grown in osteogenic media, containing Dulbecco's modified Eagle's culture (DMEM) with 10% foetal bovine serum (FBS) and an antibiotic (G480 30 mg/uL). Cells were expanded by incubation at 35 °C in 75 cm^2 flasks with 5% CO_2. Viability assays were performed for 7 days. As a negative control, prior to the labelling process, hFOB cultures were incubated with 70% methanol for 30 min. As a positive control, hFOB cultures without any treatment were used. Prior to the test, the materials were sterilised using an autoclave at 120 °C for 20 min. For the viability assay, cells were grown in a complete medium in 96-well plates at a concentration of 2×10^4 cells/well. These plates were incubated at 35 °C with 5% CO_2 for 24 h. In the case of the positive control (PC) and negative control (NC),

the wells were left to grow without adding anything, while in the test wells, the different biomaterials were added.

To measure viability, the MTS assay was used. Before labelling, as a negative control, the cells were incubated with 70% methanol for 30 min. Twenty uL of MTS was added to all wells and incubated at 34 °C for 1 h, after which absorbance was measured at 490 nm using the Varioskan LUX plate reader (Fisher Scientific S.L., Alcobendas, Spain). This process was carried out at 24 h, 48 h, 72 h, and 7 days of incubation, with the different BBC/Epoxy sample solutions. The test was performed in $n = 3$ replicates.

In addition, a LIVE/DEAD assay was carried out to determine whether the cells showed growth adhering to the surface of the tested biomaterials. For this, cells were grown on the surface of the materials under the same conditions as described above, for 24 h, after which the reagents Calcein (3.5 µM) and EthD-1 (7 µM) were added and incubated for 1 h at 37 °C. For the positive control, cells were grown without the presence of any biomaterial. For the negative control, the same materials were used, treated with the reagents, without cell culture. The biomaterials, as well as the controls, were observed under a fluorescence microscope to determine if there had been growth of adherent cells on the surface.

3. Results and Discussion

In view of the results obtained in the MTS assay (Figure 3), none of the BBC/epoxy ratios were cytotoxic to the cell line studied. The positive control, culture medium in the absence of biomaterial, was taken as 100% viability, so no significant differences were found with any of the BBC/epoxy ratios tested. The negative control, 70% methanol for 30 min prior to the test, did not exceed 40% viability in any of the cases. All tests were repeated a minimum of three times (n = 3). The ANOVA test showed that there were significant differences between samples ($F = 4.202$, $p < 0.005$).

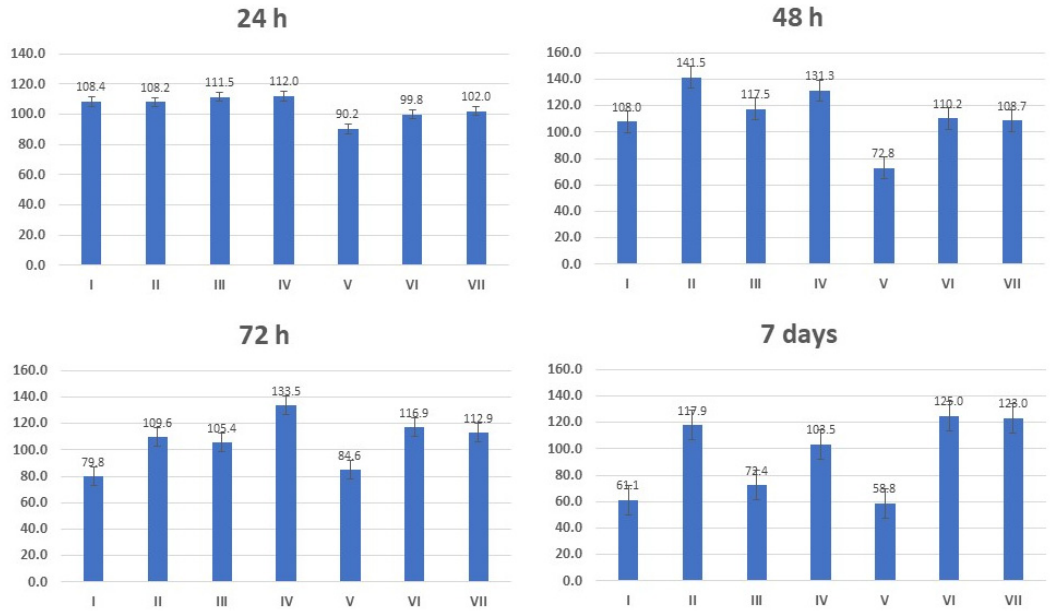


Figure 3. Viability of human osteoblasts after 24 h, 48 h, 72 h, and 7 days of incubation with the different biomaterials tested. Culture medium in the absence of any biomaterial was used as a positive control, while 70% methanol was used as a negative control. X: sample tested; Y: viability (%).

The LIVE/DEAD test, which tests the adhesion of the growing cells on the surface of the biomaterials, showed that only in the proportion in which 50% of BBC was reached was there cell growth in adhesion on the surface of the biomaterial. These results are presented in Figure 4, which shows the growth of cells on the surface of biomaterial VI, both with cells fixed with 70% methanol (a) and with live cells (b).

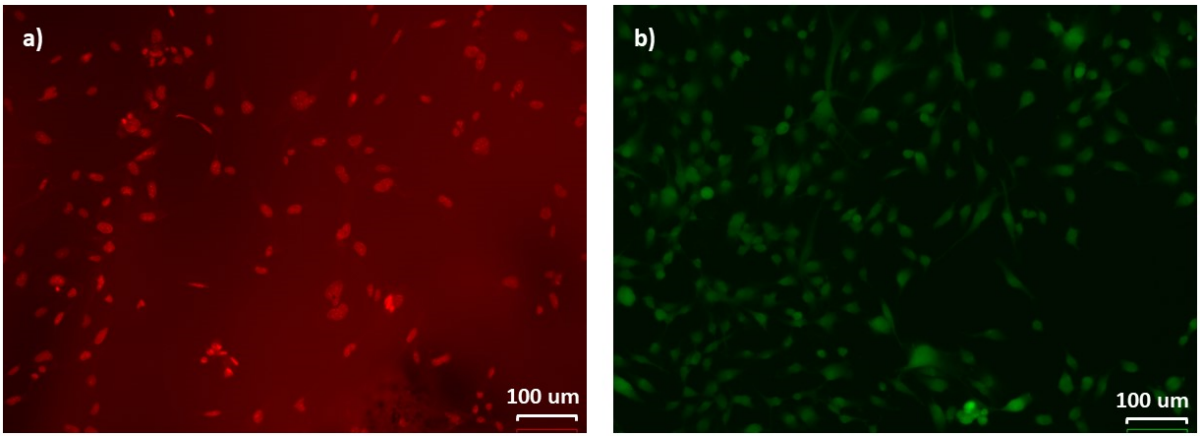


Figure 4. Growth of cells adhered to the surface of the biomaterial VI; (**a**) cells fixed with 70% methanol; (**b**) live cells.

In addition, sample VII, enriched with SBP, was also tested with the LIVE/DEAD test, showing that the cells also grew attached to the surface of the sample, the results of which are shown in Figure 5.

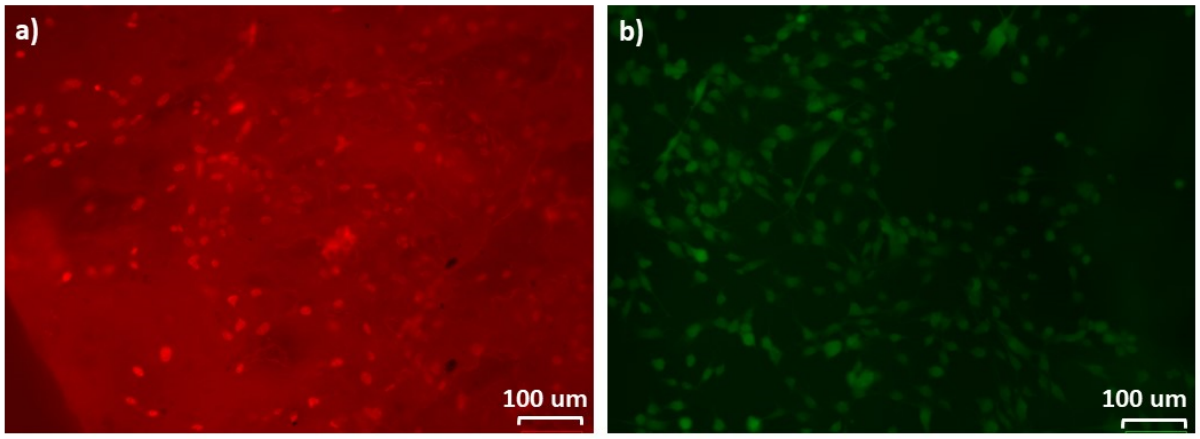


Figure 5. Growth of cells adhered to the surface of the biomaterial VII; (**a**) cells fixed with 70% methanol; (**b**) live cells.

Given that the best BBC/epoxy ratio was shown to be the one corresponding to mixture VI, a comparison of the viability results of this mixture with the sample in which SBP was included was drawn. Figure 6 shows the comparative results of both samples (VI and VII), evidencing that there were no significant differences between them. This result indicates that the addition of beet fiber to the sample, as an enrichment of the scaffold, did not affect the cell viability of osteoblasts in vitro.

These results are in line with others where natural fibres were added to HA scaffolds to improve them, showing that the composite had good cell biocompatibility and that they could be used as biomedical materials [20,45].

Figure 7 shows the results obtained from the adhesion tests of the biomaterial to the three implantable surface types. The results indicated that although all materials showed similar initial adhesion strength, U1010 maintains its adhesion over time. Ti-6Al-4V and Vitalium® experienced slight losses in adhesion.

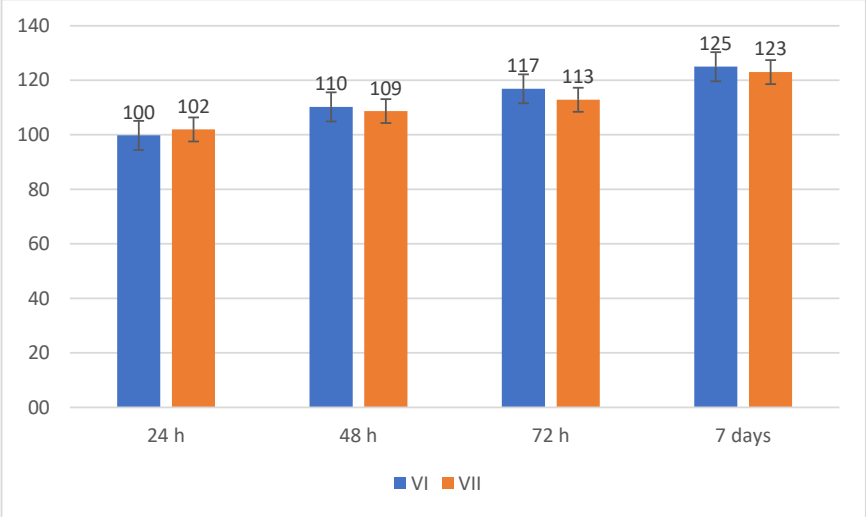
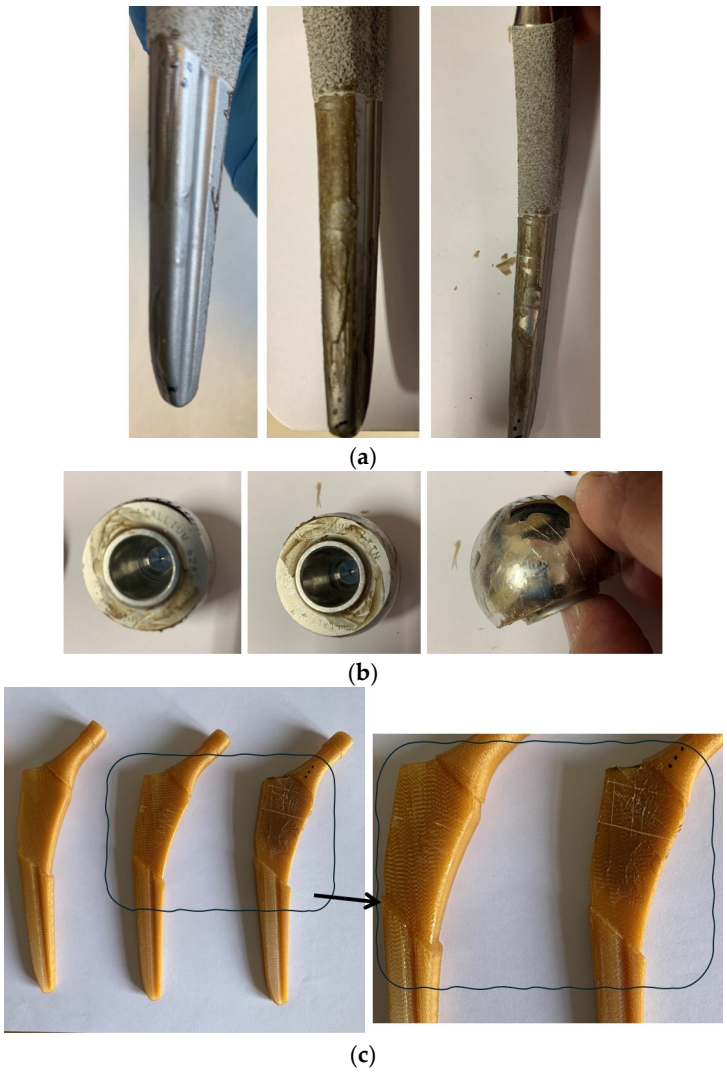


Figure 6. Viability of human osteoblasts after 24 h, 48 h, 72 h, and 7 days of incubation with samples VI and VII. Culture medium in the absence of any biomaterial was used as a positive control, while 70% methanol was used as a negative control. X: measure of time (hours/days); Y: viability (%).



(**a**)

(**b**)

(**c**)

Figure 7. Results of adhesion tests of the biomaterial to implantable surfaces; (**a**) coating applied on the distal part of the Ti5 hip prosthesis; (**b**) coating applied to the femoral head of the hip prosthesis

made of Vitalium; (**c**) coating applied on the intermediate area of the femoral stem of the hip prosthesis, manufactured in FDM 3D printing in U1010 material. In the enlarged view, two coatings can be seen, type I on the left and type VII on the right.

As seen in Figure 7a,b, Ti5 and Vitalium cause the coating to peel off more easily due to their hardness. However, as seen in Figure 7c U1010 behaves with more resilience, the thermoplastic shows greater adhesion when tested. As can be observed on the surface of the intermediate zone of the hip prosthesis, the cross-cut test makes the traces of the blades visible, with no discernible detachment to the naked eye. For this reason, it obtained a result of 0 on a scale from 0 to 5, with 0 being the optimal result according to UNE-EN ISO 16276-2:2008 [46], while coatings on metal surfaces did not exceed Level 1 due to irregular peeling of the coating along either side of the incisions, equal to or less than 1.5 mm.

4. Conclusions

Nowadays, an increasing number of surgical operations where it is necessary to use bone filler (bone cement) are performed. This commercial material is composed of This commercial material is composed of a resin- and mineral-based composite of bovine origin. This study conducted on the mixture of BBC and an epoxy-resin-based adhesive yielded promising outcomes regarding its interaction with bone cells. In our research, we observed that the mixture did not affect the proliferation of bone cells. Furthermore, it was determined that the surface of this mixture served as a surface for the growth of bone cells, indicating its potential for fostering bone tissue development. These findings suggest favourable biocompatibility between the BBC/Epoxy resin mixture and bone cells, highlighting its promising role in promoting bone cell proliferation and growth. This could have significant implications for various biomedical applications, particularly in bone tissue engineering and regenerative medicine. The adhesion test performed on three different implantable surfaces, U1010, Vitallium, and Ti5, showed that there was no significant detachment, thus highlighting the result obtained by U1010 compared to the other two, having used the coating based on the BBC/SBP VII hybrid sample. The method of application for implantable elements is suggested in the form of a single vial, in which the compound is already mixed in the appropriate proportions.

Author Contributions: Conceptualization, M.S. and M.Á.C.-L.; methodology, M.Á.C.-L., C.R. and P.A.-C.; formal analysis, M.S. and C.P.-M.; investigation, C.P.-M., M.S. and D.G.-J.; resources, M.S.; writing—original draft preparation, M.S., C.P.-M. and D.G.-J.; writing—review and editing, M.S. and M.Á.C.-L.; supervision, M.Á.C.-L., C.R. and P.A.-C.; project administration, M.S.; funding acquisition, M.S. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original contributions presented in this study are included in the article Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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