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(71) Applicants (for all designated States except US): FUN-

DAÇÃO DE AMPARO À PESQUISA DO ESTADO
 DE SÃO PAULO [BR/BR]; Rua Pio XI, 1500, Alto da
 Lapa, 05468-901 São Paulo - SP (BR). UNIVERSI-
 DADE ESTADUAL PAULISTA JULIO DE

MESQUITA FILHO [BR/BR]; Rua Quirino de An-
 drade, 215, Centro, 01049-010 São Paulo - SP (BR).

(72) Inventors; and

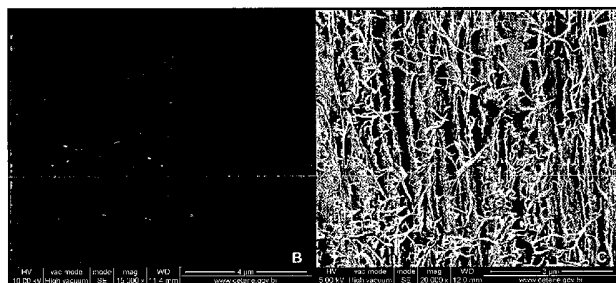
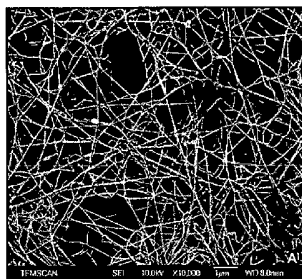
(75) Inventors/Applicants (for US only): SASKA, Sybele
 [BR/BR]; Rua Luiz Saska, 134, Jardim Celiamar,
 14806-153 Araraquara - SP (BR). MARCHETTO,
 Reinaldo [BR/BR]; Rua Padre Duarte, 985, Apto. 23 -
 Centro, 14801-310 Araraquara - SP (BR). MESSAD-
 DEQ, Younès [BR/BR]; Avenida Doutor Agostinho Tuc-
 ci, 1122, Vila Harmonia, 14802-590 Araraquara - SP
 (BR). RIBEIRO, Sidney José de Lima [BR/BR]; Rua
 Doutor Clemente Ferreira, 1135, Jardim Marivan,
 14806-152 Araraquara - SP (BR). GASPARG, Ana Maria
 Minarelli [BR/BR]; Avenida Benito Barbieri, 805, Vila
 Harmonia, 14802-570 Araraquara - SP (BR).

(74) Agents: SANTOS CELIDONIO, Ana Paula et al.;
 Avenida Brigadeiro Faria Lima, 1485, 11º andar - Torre
 Norte, 01452-002 São Paulo (BR).

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(54) Title: METHODS FOR OBTAINING REABSORBABLE COMPOSITES, COMPOSITES, MEMBRANE, SCAFFOLD
 AND ITS USES



SEM micrographs : A = CB membrane; B = CB-COL membrane; C = cross section of the CB-
 COL membrane.

FIGURE 5

(57) Abstract: The present invention relates to novel
 methods of obtaining reabsorbable composites, based on
 bacterial cellulose and collagen, for application in tissue
 repairing. Additionally, the present invention relates to the
 composites obtained by the methods described herein and
 their uses.



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**“METHODS FOR OBTAINING REABSORBABLE COMPOSITES,
COMPOSITES, MEMBRANE, SCAFFOLD AND ITS USES”**

FIELD OF THE INVENTION

The present invention relates to novel methods of obtaining
5 reabsorbable composites, based on bacterial cellulose and collagen, for
application in tissue repairing.

Additionally, the present invention relates to the composites
obtained by the methods described herein and their uses.

BACKGROUND OF THE INVENTION

10 The development of biomaterials is fundamentally important once
it is related to an improvement in quality of life, represented by an increase in
life expectancy, in health in general and in the population's welfare. In this way,
it is observed a huge effort to produce new biomaterials devices for medical
application.

15 In the clinical practice, it is extremely important that the
development of materials for tissue replacement/ repairing application be based
on the perfect knowledge of the structures to be replaced. Ideally, a
replacement material should imitate the living tissue from the mechanical,
chemical, biological and functional viewpoints. The current trend is an attempt
20 to regenerate the tissue instead of replacing it. This approach requires the use
of biodegradable polymers with specific properties. So, research efforts focused
on the development of new reabsorbable systems for clinical application are
increasing.

25 Different types of reabsorbable membranes have been used for
filling of surgical failures, bone defects or protection of the lateral wall of the
maxillary sinus, being performed in order not to show any rejection signs, and
for others indications such as in the wounds' protection acting as a biological
curative.

30 These membranes create a propitious way of improving the
functional repairing through the natural biological potential, which includes the
following factors: blood clot mechanical stability, creation and maintenance of its
space and isolation of undesirable tissues from the repairing place.

The vegetable cellulose has been largely used in the field of

biomaterials, such as hemostatic agents (Valentine, R.; Wormald, P.J.; Sindwani, R.; *Advances in absorbable biomaterials and nasal packing*. Otolaryngol Clin North Am. (2009) 42: 813-28), however, its production involves well-honed extraction and purification processes.

5 The bacterial cellulose is becoming an alternative to the application in the production of biomaterials, as it has numerous characteristics that make it different from the vegetable cellulose, such as higher crystallinity, purity, traction resistance, elasticity, durability, high capacity for water absorption and retention besides ultraviolet radiation absorption (Klemm, D.; Heublein, B.; Fink, H-P.; Bohn, A. Cellulose: fascinating biopolymer and sustainable raw material. *Angew. Chem. Int. Ed.* (2005) 44: 3358 – 3393).

10 In this aspect, it is known in the art the use of bacterial cellulose in conjunction with collagen for application in biomaterials (Wiegand, C.; Elsner, P.; Hippler, U.C.; *et al.* Protease and ROS activities influenced by a composite of bacterial cellulose and type I collagen *in vitro*. *Cellulose* (2006) 13: 689–696; Honglin, L.; Xiong, G.; Huang, Y.; He, F. *et al.* Preparation and characterization of a novel COL/BC composite for potential tissue engineering scaffolds, *Materials Chemistry and Physics* (2008) 110: 193–196), wherein the collagen is added to the culture medium for the *Acetobacter xylinum*. In this case, the collagen was
20 incorporated into the cellulose in the production process of cellulose fibers by the bacterium, which restricts the uniformity in terms of amount of collagen incorporated into cellulose fibers, which does not occur covalently. Thus, the homogeneity of the composite formed is questionable due to the fact that the amount of collagen is predisposed towards the metabolism of bacteria, making it impossible to state
25 whether there are standardization of the collagen content in the composite formed on these methodologies. This standardization is crucial, since the ultimate goal of these products is the *in vivo* application.

 Therefore, considering the importance of such composite in the current medical technology and the disadvantages presented by methods
30 known in the art, there is a growing need for alternative, more effective and economically viable composites.

SUMMARY OF THE INVENTION

 Thus, the present invention provides a novel method of

reabsorbable composites production based on bacterial cellulose and collagen, which promotes a more effective tissue repairing, wherein the collagen is covalently incorporated into the matrix by cross-linking and homogeneously, which allows the control of the amount of collagen incorporated and consequently the increase of this protein's concentration inside the cellulose matrix.

The methodology employed by the present invention has made it possible to design a more compact and homogeneous system with the filling pores, an important characteristic for a membrane to be employed as a mechanical barrier, because will further improve the function of cell occlusion.

According to the present invention, the method comprises the following steps:

- a) obtaining of a bacterial cellulose;
- b) Esterification of an amino acid or an amino acid derivative to the membrane of bacterial cellulose, using as activating agents the carbonyldiimidazole (CDI) and N-methylimidazole (NMI); and
- c) Incorporation of collagen with the proviso that when one amino acid derivative is used, such incorporation is preceded by deprotection of the amine group from the amino acid derivative incorporated into the membrane.

According to the present invention, the method may include the further step of precipitation and incorporation of the hydroxyapatite (HAp) after step (c).

Preferably, according to the present invention, the method comprises the subsequent incorporation of peptides that induce the proliferation and/or cell differentiation.

Another particularly preferred object of the present invention is the reabsorbable composite obtained by the methods of the present invention.

Additionally, the use of the composite obtained by the methods described herein for tissue repairing, especially for bone tissue regeneration and healing or regeneration of connective tissue, is also object of the present invention.

Another embodiment of the present invention relates to a reabsorbable composite comprising, respectively, bacterial cellulose, collagen

and hydroxyapatite, and their uses in bone tissue regeneration.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the primary structure of peptides OGP and OGP (10-14).

Figure 2 is a schematic representation of the Erlenmeyer flask used to culture the bacteria *Acetobacter xylinum* and subsequent production of hydrated bacterial cellulose pellicle.

Figure 3 shows the analytical-scale chromatogram of the OGP (10-14) peptide.

Figure 4 shows the analytical-scale chromatogram of the OGP of the OGP peptide (a - crude b - purified).

Figure 5 depicts SEM micrographs showing in A – BC membrane, B - BC-COL membrane, C - cross section of the BC-COL membrane.

Figure 6 shows the XRD diffraction patterns of (a) BC, (b) COL, (c) BC-COL with their peaks marked.

Figure 7 represents the vibrational spectrum in the infrared region from samples: BC (a) pure collagen (b) BC-glycine (c) and BC-COL composite (d).

Figure 8 represents the vibrational spectrum in the infrared region between 1000 and 1850 cm^{-1} obtained from samples: BC (a) pure collagen (b) BC-glycine (c) and BC-COL composite (d) showing the displacement to the lower energy region.

Figure 9 depicts SEM micrographs showing: A - BC membrane, B and C - nanocomposite (BC-COL)-HAp with their respective magnifications, 2.000x and 30.000x, D and E - nanocomposite (BC-COL)-HAp in cross section with their respective magnifications 7.000x and 15.000x.

Figure 10 illustrates the P and Ca ions mapping of the (BC-COL)-Hap composite.

Figure 11 depicts the TEM image of the (BC - COL)-Hap composite.

Figure 12 represents the XRD diffraction patterns of (a) BC, (b) COL, (c) BC-COL and (d) (BC-COL)-HAp.

Figure 13 represents the vibrational spectrum in the infrared

region obtained from samples: (a) BC, (b) COL, (c) BC - COL, (d) (BC-COL)-HAp.

Figure 14 shows the epifluorescence of the osteoblastic cells derived from rat calvaria cultured on BC (A), BC-COL (B) and BC_OGP 10^{-9} M (C). The green fluorescence indicates actin cytoskeleton staining. It is noted smaller cell population of cultures on BC. Scale Bar: 100 μ m for A-C.

Figure 15 shows the epifluorescence of the osteoblastic cells derived from rat calvaria cultured on BC (A), BC/COL (B), BC_OGP 10^{-9} M (C), BC_OGP 10^{-6} M (D), BC_OGP (10- 14) 10^{-6} M (E) and BC_OGP (10-14) 10^{-9} M (F). The green fluorescence indicates actin cytoskeleton staining. Scale Bar: 100 μ m for A-C.

Figure 16 shows the epifluorescence of the osteoblastic cells derived from rat calvaria cultured on (BC-COL)-HAp (A), (D) and (G), (BC-COL)-HAp_OGP 10^{-9} M (B), (E) and (H) and (BC-COL)-HAp_OGP (10-14) 10^{-9} M (C), (F) and (I). Green fluorescence indicates actin cytoskeleton staining and red fluorescence indicates nuclear DNA staining.

Figure 17 depicts the cellular proliferation of osteoblastic cells derived from rat calvaria cultured on BC and (BC-COL)-Hap membranes associated or not with growth factors, assessed at 14 and 21 days. Data are presented as mean $\pm$ standard deviation.

Figure 18 depicts the alkaline phosphatase (ALP) activity of osteoblastic cells derived from rat calvaria cultured on BC and BC (BC-COL)-Hap membranes associated or not with growth factors, assessed at 21 days. Data are presented as mean $\pm$ standard deviation.

DETAILED DESCRIPTION

In one embodiment, the present invention relates to a method of obtaining reabsorbable composites based on bacterial cellulose and collagen, which comprises the following steps:

- a) obtaining of bacterial cellulose;
- b) Esterification of an amino acid or an amino acid derivative to the membrane of bacterial cellulose, using as activating agents the cabonyldiimidazole (CDI) and N-methylimidazole (NMI); and
- c) Incorporation of collagen with the proviso that when one amino

acid derivative is used, such incorporation is preceded by deprotection of the amine group from the amino acid derivative incorporated into the membrane.

According to an embodiment of the invention, the step (a) can be made by any method for obtaining bacterial cellulose known in the prior art. In a particular embodiment, the bacterial cellulose of the present invention is obtained by culturing the *Acetobacter xylinum* bacteria in static culture medium.

The step (b) of the method above is accomplished by modifying the surface of the bacterial cellulose matrix by esterification of an amino acid or amino acid derivative, as described by Hilpert *et al.* (Hilpert, K.; Winkler, D.F.H.; Hancock, R.E.W. Peptide arrays on cellulose support: SPOT synthesis, a time and cost efficient method for synthesis of large numbers of peptides in a parallel and addressable fashion. *Nature Protocols*. (2007) 2: 1333-1349).

Methods of esterification using carbodiimides as activating agents are well known in the art, and due to the low speed of reaction, catalysts such as Dimethylaminopyridine (DMAP) are employed. However, due to the basic character of such catalyst, a variety of unwanted reactions may occur, such as the high rate of racemization and formation of dipeptides or oligomers, which are dependent on a number of factors, including the nature of the amino acid or amino acid derivative and reaction time.

Therefore, the present invention uses a different method of esterification, which overcomes the disadvantages described above. According to the present invention, the esterification carried out uses the carbonyldiimidazole (CDI) as carboxylate-activating agents of the amino acid or amino acid derivative, whose reactivity is similar to the acid chlorides used in such technique, however, more easily manipulated. In addition, in the esterification of the present invention, it is required the presence of a potent nucleophile, which acts as a catalyst, a role played by N-methylimidazole (NMI).

The NMI, along with CDI, provides very good yield and side products such as carbon dioxide and imidazoles, are relatively innocuous. Furthermore, the racemization of amino acids or amino acid derivatives also tends to be minimal, due to the mild conditions of reaction, which does not occur with other esterification mechanisms. Additionally, the reaction time employed by the esterification of the present invention is shortened (maximum

of 2 hours) and does not allow the formation of oligomers as in the case of the use of DMAP, which employs a reaction time of about 3 hours.

In one particular embodiment of the present invention, the amino acid or amino acid derivative in step (b) is aliphatic and nonpolar. More particularly, the amino acid is glycine (Gly) and amino acid derivative is 9-Fluorenylmethyloxycarbonyl -glycine (Fmoc-Gly).

After the completion of step (b), the measurement of the degree of esterification is performed by UV spectrophotometry at 290 nm. After assessing the degree of amino acid or amino acid derivative incorporation into bacterial cellulose, the following step (c) of collagen insertion is conducted.

In a particular embodiment of the present invention, the step (c) of collagen incorporation comprises the following steps:

c1) deprotecting the amino group from amino acid derivative incorporated into the membrane when an amino acid derivative is used; and

c2) performing a covalent bond between the amino acid or amino acid derivative covalently bound to the matrix and the collagen that will be inserted, using an aqueous solution containing collagen (1 eq) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (4 eq) at a temperature of 0 °C to 10 °C, preferably at 4 °C.

The amount of collagen in the system is measured by hydroxyproline analysis using the Woessner's method (Woessner Jr., J.F. The Determination of Hydroxyproline in Tissue and Protein Samples Containing Small Proportions of this Imino Acid. *Arch Biochem Biophys.* 1961; 93:440-447).

According to the present invention, the method may further comprise the additional step of precipitation and hydroxyapatite (HAp) incorporation following the step (c). Particularly, such additional step is accomplished by:

- incorporating hydroxyapatite by methods known in the art. (Hutchens, A.S.; Benson, R.S.; Evans, B.R.; *et al.* Biomimetic synthesis of calcium-deficient hydroxyapatite in a natural hydrogel. *Biomaterials.* 2006; 27: 4661-4670); and

- immersing the composite cellulose-aa-collagen first in a solution containing calcium and then in solutions containing phosphate.

All cycles of incubation for HAp incorporation are performed preferably at room temperature for 24 hours.

In a particular embodiment, according to the present invention, the method comprises a subsequent incorporation of peptides that induce the proliferation and/or cell differentiation, wherein the composites obtained, with
5 HAp incorporation or not, are maintained in solution containing said peptides.

In particular, such peptides are incorporated into the cellulose matrix through adsorption. The potential peptides to be used may have various purposes according to their primary structure, and they may be modulators or
10 growth factors and antimicrobials.

In a preferred embodiment of the present invention, the peptides concentration in solution is in the range of 10^{-6} to 10^{-9} M.

According to the present invention, the peptides that can be used are inducers of cellular differentiation and proliferation. Particularly, the peptides
15 are OGP (osteogenic growth peptide) or OGP (10-14) (osteogenic growth peptide (10-14)).

In another embodiment, the composites obtained by the method of the present invention may be subsequently:

- dried at 37 °C for the manufacture of membranes for tissue
20 repairing, or
- freeze-dried for making scaffolds.

In another aspect, the present invention relates to composites obtained by the methods described herein.

Another particularly preferred object of the present invention
25 relates to the use of composites obtained by the methods described herein for tissue repairing. By "tissue repairing" it is meant applications such healing or regeneration of any tissue type. Particularly, the composite of the present invention can be used to regenerate a bone tissue and repair of dermal or epidermal tissue.

30 Therefore, the present invention preferably relates to use of said composite for bone tissue regeneration and tissue repair of the epidermis and dermis.

In another embodiment, the present invention relates to a

reabsorbable composite comprising, respectively, bacterial cellulose, collagen and hydroxyapatite, and their uses for bone tissue regeneration.

Particularly, the bacterial cellulose contained in the composite of the invention is obtained from any bacterial source known in the art. Preferably, the bacterial cellulose is obtained by growing the bacteria *Acetobacter xylinum* in static culture medium.

The present invention can be more fully and clearly understood from the following examples, which are offered for illustrative purposes only and are not intended to limit the scope of the present invention in any way.

EXAMPLES

EXAMPLE 1

1 - CHEMICAL SYNTHESIS OF THE OGP AND OGP (10-14)

It was performed the synthesis of two peptides in order to elucidated which one would be most effective for cell proliferation and differentiation.

Thus, the peptides OGP and OGP (10-14) derivative thereof (Figure 1) were synthesized manually by solid phase method (STEWART, J. M.; YOUNG, J. D. In: Merrifield RB. (Ed.) *Solid phase peptide synthesis*: 2^a Ed., New York: Pierce Chemical Company, 1984; LLOYD-WILLIAMS, P.; ALBERICIO, F.; GIRALT, E. *Chemical approaches to the synthesis of peptides and proteins*. Boca Raton: CRC, 1997; AMBLARD, M.; FEHRENTZ, J. A.; MARTINEZ, J.; SUBRA, G. *Fundamentals of modern peptide synthesis*. Methods Mol. Biol., v. 298, p. 3-24, 2005), according to standard protocol employing the base labile 9-Fluorenylmethyloxycarbonyl (Fmoc) group for protection of α -amino groups, and t-butyl (t-Bu) derivatives for protection of most side chains of trifunctional amino acid residues (FIELDS, G. B.; NOBLE, R. L. Solid phase peptide synthesis utilizing 9-fluorenylmethoxy-carbonyl amino acids. *Int. J. Pept. Protein. Res.*, v. 35, p. 161-214, 1990; CHAN, W. C.; WHITE, P. D. *Fmoc solid phase peptide synthesis: a practical approach*. New York: Oxford University Press, 2000). It was started from 500 mg of a Fmoc-Gly-Wang resin (substitution degree of 0.66 mmol/g) (Novabiochem ®) and DIC/HOBT (Fluka ® and Advanced Chem Tech®, respectively) as condensing agents. In the synthetic route after coupling the fifth amino acid residue (Tyr),

the peptide resin obtained was divided into two fractions - one of them was stored for future deprotection and cleavage and the other one continued with the synthesis of the sequence of OGP, as illustrated in Figure 1. The side chain protecting groups from Fmoc-amino acids are shown in Table 1. The Fmoc-amino acids used in synthesis were purchased from Novabiochem ® or
 5 Advanced Chem Tech ®.

TABLE 1. AMINO-PROTECTING GROUPS USED IN THE SYNTHESIS OF OGP AND OGP (10-14)

Fmoc-amino acids	Acronym	side-chain protecting group
Arginine	Arg, R	pmc
Glutamine	Gln, Q	trt
Lysine	Lys, K	boc
Tyrosine	Tyr, Y	tbu
Treonine	Thr, T	tbu

In the condensation step of each amino acid, it was used a molar
 10 excess compared to the initial substitution degree of 3 equivalents for each Fmoc-amino acid and 3 equivalent for each DIC and HOBt, in a DCM:DMF (1:1) mixture (DCM Synth®, and DMF Synth®). A solution of piperidine 20% (v/v) (Merck ®) in DMF was employed in the α -amino deprotecting procedure (removal of the Fmoc group).

15 The condensation step efficiency was monitored by Kaiser's test (KAISER, E.; COLLESCOTT, R.L.; BOSSINGER, C.D.; COOK, P.I. Color test for detection of free terminal amino groups in solid-phases synthesis of peptides. *Anal Biochem.*, v. 34, n. 2, p. 595-598, 1970), and, if positive (presence of free amino groups), the process was repeated with 50% of the
 20 initial reagents amount.

The final cleavage of peptides from their resins and the deprotection of the side chain protecting groups were carried out with a solution containing 94.5% TFA, 2.5% EDT, 0.5% Tioanisol and 2.5% ultra-pure water at 25 °C for 2 hours. Reagents of this solution were purchased from Fluka®,
 25 Acros® and Sigma®, respectively; ultrapure water was obtained from Barnstead filtration system, equipped with cartridges for salts and organic compounds retention. The crude OGP and OGP (10-14) peptides were precipitated and washed with diethyl ether cooled and centrifuged (six times), dissolved in

aqueous solution of acetic acid 10%, concentrated and lyophilized.

Finally, the isolated products were analyzed and purified by high performance liquid chromatography (HPLC) in analytical scale and semi-preparative modes, respectively.

5 The proportion of amino acids, as well as synthesis yield and peptide content of the sequences synthesized and purified were determined by amino acid analysis and mass spectrometry (ESI-MS positive mode).

1.1 - HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

1.1.1 – ANALYTICAL MODE

10 The analytical scale high efficiency liquid chromatography was performed in a Varian ProStar instrument equipped with a C18 reverse-phase column. The system comprises two model 210 pumps, ProStar 400 autosampler, ProStar 320 UV/Visible Detector model 320 and a Star integrator software, all controlled by a workstation for data manipulation and processing. All the solvents
15 were chromatographic grade and all water used was ultrapure type.

 The chromatographic conditions were as follows:

 - column: a C18 reverse-phase column, Jupiter-Phenomenex (250 x 4,6 mm); d = 5 µm; 300 Å.

 - Solvents: A – Ultra-pure water containing 0.045% TFA;
20 B – Acetonitrile (Tedia) containing 0.036%
TFA

 - gradient: 5 to 95% B for 30 minutes

 - flow rate: 1.5 mL/min

 - detector wavelength: 220 nm

1.1.2 - SEMI-PREPARATIVE MODE

25 The semi-preparative high efficiency liquid chromatography was performed on a Beckman System Gold equipped with a C18 reverse-phase column. The system comprises two model 116 pumps, UV/Visible Detector connected to a Chart Recorder from Amersham Biosciences Model: REC112. All the solvents were
30 chromatographic grade and all water used was ultrapure type.

 The chromatographic conditions were as follows:

 - column: a C18 reverse-phase column, Delta Pack – Waters (300 x 19 mm); d = 15µm; 300 Å

- Solvents: A – Deionized water containing 0.045% TFA;
B – Acetonitrile containing 0.036% TFA
- gradient: 25 to 45% B for 120 minutes
- flow rate: 5 mL/min
- detector wavelength: 220 nm

1.2 – AMINO ACIDS ANALYSIS

Peptides were previously weighed (approximately 1 mg) and hydrolyzed in 1 mL of 6M HCl and 160 mL of 5% phenol in water at 110 °C for 72 hours in a N₂ atmosphere under stirring. After hydrolysis, the material was concentrated under vacuum, then dissolved in dilution buffer 0.2 M sodium citrate, pH 2.2 and filtered through Millex GV unit (0.22 µm) (Millipore ®) before injection into the HPLC system.

The amino acids analysis were performed by Shimadzu Liquid Chromatograph equipped with three LC-10A/C-47A pumps, one SIL 10AF autosampler, UV SPD 10A and fluorescence RF 10A detectors, employing the post-column method using ortho-phthalaldehyde (OPA). The system is periodically calibrated with a standard mixture of amino acids, yielding a value for elution time of each amino acid and the conversion factor between peak area and sample concentration.

To calculate the relative ratio of amino acids of the samples the ratio between their unit concentrations and the average was determined.

1.3 - MASS SPECTROMETRY

Analyses were performed by liquid chromatography mass spectrometry (LC/ESI) from samples of pure peptides, for confirmation and identification of peaks with molecular weight corresponding to the theoretical values thereof. The LC/ESI system used was from Waters-Micromass, comprising an Alliance model 2690 separation module, 996 Photodiode Array Detector, a 120 vial capacity autosampler and a Micromass model ZMD mass spectrometer. The system is controlled by a Compaq Workstation AP200.

2 - PREPARATION AND CHARACTERIZATION OF ORGANIC-INORGANIC COMPOSITES

2.1 - PREPARATION OF BACTERIAL CELLULOSE MEMBRANES

The bacterial cellulose (BC) pellicle were obtained by growing *Acetobacter xylinum* (overproducing strain) in static culture medium, which

contained the following basic composition: 2% (w/v) glucose, 0.5% (w/v) peptone, 0.5% yeast extract, 0.27 % (w/v) and disodium phosphate anhydrous 0.115% (w/v) citric acid monohydrate.

The bacterial culture was performed in a 500-ml Erlenmeyer flask for 120 hours of culturing time at 28 °C (5 mm thick) (Figure 2). In the static culture medium, the cellulose was obtained in the form of a gelatinous membrane formed on the medium/surface interface. To remove the bacteria, the membrane has been undergone a treatment with dilute hydroxide and sodium hypochlorite solution for 30 minutes, then exhaustively washed with distilled water and stored in ultra-pure water. After several changes of ultrapure water, the membranes were autoclaved for 15 minutes at 120 °C.

2.2 PREPARATION OF COMPOSITES

The membranes developed were based on bacterial cellulose, type I collagen, hydroxyapatite and growth factor (Table 2).

For the production of such membranes, the highly hydrated BC matrix with 5 mm thickness was prepared with 13 and 25 mm diameters.

TABLE 2 - COMPOSITION OF THE MADE COMPOSITES

Membranes	Membranes / OGP	Membranes / OGP [10-14]
BC	BC + OGP	BC + OGP [10-14]
BC - COL	(BC-COL) + OGP	(BC - COL)+ OGP [10-14]
(BC-COL)- HAp	(BC-COL)- HAp + OGP	(BC-COL)- HAp + OGP [10-14]

2.2.1 - PREPARATION OF BACTERIAL CELLULOSE AND COLLAGEN COMPOSITES

A solution of type I collagen from rat tail tendon (concentration 4 mg/mL) (Sigma®-Saint Louis/USA) was used for preparing highly hydrated BC matrix with 5 mm thick and 25 mm in diameter.

To perform the process of collagen incorporation, firstly the water of BC was changed by DMF (Synth®). This change was performed by vacuum filtration in porous plate filter. After the completion of the change the surface modification of BC by esterification of an Fmoc-amino acid (Advanced Chem Tech®) was performed to the free hydroxyl groups of cellulose

To this end, the following reagents were used: 0.1 M Fmoc-amino acid, 162mg 1,1'-carbonyldiimidazole (CDI) (Sigma®), 127 µL N-methylimidazole (NMI) (Fluka®) in 8 mL DMF. The reaction time was 2 hours at room temperature under stirring.

After the reactions, several washes with DMF under vacuum filtration and subsequent immersion in DMF for 24 hours for complete removal of excess reagents have been carried out.

Quantitative analysis by spectrophotometry in ultraviolet-visible spectrophotometer (UV-VIS) Shimadzu UV-1601PC was used to determine the degree of Fmoc-amino acid incorporation to the OH groups of BC. The absorbance of the samples was measured using a wavelength of 290 nm.

To perform these measures, a small fraction of each sample was weighed and then treated with 20% (v/v) piperidine in a quartz cuvette (10 x 10 mm). After shaking, the absorbance reading was performed against a blank where the sample has been omitted. The degree of incorporation (DI) of the amino acid derivative was calculated using the equation below:

$$GI \text{ (mmol/g)} = \frac{A \text{ sample} - A \text{ reference (mg)}}{1.65 \times \text{sample weight (mg)}}$$

Deprotection of the α -amino groups was performed with 20% (v/v) piperidine solution for 2 hours at room temperature was proceeded. After the deprotection-processing, successive washes with DMF were performed under a vacuum system to remove the excess of the piperidine solution, followed by a series of washes with DMF and subsequent repeated washing with ultrapure water for solvent exchange, and then the collagen incorporation to BC matrix was performed.

To this end, it was used 600 μ L of type I collagen solution (4 mg/mL) and 5 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Novabiochem ®) in 10mL of ultra-pure water. Subsequently, the BC samples were immersed in this modified solution by adjusting the initial pH at 6.0 with 0.2 M NaOH and maintaining in this condition for 24 hours at 4 °C. After this period, the samples were washed with ultrapure water under vacuum to remove the collagen and EDC excess.

BC-COL samples thus obtained were dried in a pressed mold at 37 °C and placed in envelopes suitable to gamma radiation sterilization (25kGy sterilization dose). BC-COL samples which will be used for subsequent incorporation of growth factor were stored at 4 °C. The BC-COL samples were frozen and lyophilized for scaffolds production.

2.2.2 PREPARATION OF BACTERIAL CELLULOSE, COLLAGEN AND HYDROXYAPATITE

((BC-COL)-HAP) COMPOSITES

To obtain such composites, the collagen was initially incorporated into the BC, according to the protocols BC-COL described in item 2.2.1, and then HAp.

5 The hydroxyapatite incorporation was carried out by methods known in the art (Hutchens, A.S.; Benson, R.S; Evans, B.R.; *et al.* Biomimetic synthesis of calcium-deficient hydroxyapatite in a natural hydrogel. *Biomaterials*. 2006; 27: 4661-4670).

10 Afterwards, the immersion of cellulose-aa-collagen composite was performed firstly in 0.05M CaCl₂ solution (calcium chloride) and subsequently in a 0.1 M Na₂HPO₄ solution (sodium hydrogen phosphate).

All cycles of incubation for HAp incorporation were performed at room temperature for 24 hours.

15 Likewise, the (BC-COL)-HAp samples were dry into pressed mold at 37 °C, and the membranes thus obtained were placed in envelopes to gamma radiation sterilization (25kGy sterilization dose). The procedures for subsequent growth factors incorporation were the same as those described for BC-COL. The (BC-COL)-HAp samples were frozen and lyophilized for scaffolds production.

2.2.3 OGP AND OGP (10-14) INCORPORATION INTO THE SAMPLES

20 The growth factor incorporation was performed by the adsorption process. By this process, the peptide binds to the composite by hydrogen bonds, whose release occur with increasing ionic strength (biological conditions). The concentrations used were 10⁻⁶ and 10⁻⁹ M. According to Spreafico *et al.* (Spreafico, A.; Frediani, B.; Capperucci, C.; Leonini, A.; Gambera, D.; Ferrata, P.; Rosini, S.; Di Stefano, A.; Galeazzi, M.; Marcolongo, R. Osteogenic growth peptide effects on primary human osteoblast cultures: potential relevance for the treatment of glucocorticoid-induced osteoporosis. *J. Cell Biochem.*, v. 1, p. 1007-1020, 2006), concentrations less than 10⁻¹⁴ showed marked cellular proliferation, whereas the concentration of 10⁻⁹ M improved the activity of alkaline phosphatase, and increased
30 bone-nodule formation and mineralization.

For this procedure, samples (13 mm diameter) were obtained following the protocols described above for BC, BC-COL and (BC-COL)-HAp. The solutions of the respective peptides and their concentrations were prepared

according to Table 3. Each sample was immersed in 5 mL of the respective solution for 72 hours at 10 °C.

TABLE 3. SOLUTIONS OF THE RESPECTIVE PEPTIDES, THEIR CONCENTRATION AND SAMPLES TYPES IN WHICH THEY HAVE BEEN INCORPORATED

Solution	Peptide	Concentration	Sample
I	OGP	10×10^{-6} M	BC, BC-COL
II	OGP	10×10^{-9} M	BC, BC-COL, (BC-COL)-HAp
III	OGP (10-14)	10×10^{-6} M	BC, BC-COL
IV	OGP (10-14)	10×10^{-9} M	BC, BC-COL, (BC-COL)-HAp

3. CHARACTERIZATION OF COMPOSITES

In the characterizations of the BC-COL and (BC-COL)-Hap products, with or without adsorbed peptides, the following techniques were employed: scanning electron microscopy (SEM), energy dispersive X-ray (EDX), transmission electron microscopy (TEM), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR) and hydroxyproline quantification.

3.1 SCANNING ELECTRON MICROSCOPY (SEM)

The SEM micrographs were taken to verify the fine structure of the samples surface morphology. The micrographs were obtained with an electron microscope model XL 30 FEG (Philips) at an accelerating voltage of 5 kV. Each sample was placed in copper support, cover with a 1 nm thick layer of gold for 60 seconds, operated at 3KV and current 9.5 μ A. Analyses were performed at the Materials Characterization Laboratory of Department of Materials Engineering at the Federal University of São Carlos - SP.

3.2 ENERGY DISPERSIVE X-RAY SPECTROSCOPY (EDX)

The microanalyses were performed in a FEG XL 30 (Philips) equipment at 5 kV to calculate the Ca/P molar ratio of the (BC-COL)-HA nanocomposites. Analyses were performed at the Materials Characterization Laboratory of Department of Materials Engineering at the Federal University of São Carlos - SP.

3.3 TRANSMISSION ELECTRON MICROSCOPY (TEM)

Transmission electron microscopy analyses were performed using

a FEI-Tecnai20 microscope at the CETENE-Recife/PE, operated at 200 kV. The samples were previously prepared by grinding and suspending in ethanol. After homogenization of the samples, a drop of suspension was deposited on the copper support.

3.4 X-RAY DIFFRACTOMETRY (XRD)

The X-rays diffraction patterns were obtained using a Kristalloflex-Siemens diffractometer with Ni filter and Cu K α radiation at 4° to 70° to determine the crystal structure and the percentage of the crystalline fraction from samples.

3.5 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

The FTIR spectra were obtained using a 8300-SHIMADZU FT-IR spectrometer. The respective samples were previously weighed and tablets were prepared with KBr mixture (1:100). Thirty- two scans were accumulated with a resolution of 4 cm⁻¹, and the results were used to identify functional groups.

3.6 DETERMINATION OF COLLAGEN BY HYDROXYPROLINE ANALYSIS

It was used the hydroxyproline quantification analysis to try to elucidate the collagen ratio incorporated in the samples. This analysis allowed determining the percentage of collagen present in each protein-containing composite.

For this purpose, the total collagen content in the samples was determined using the methodology described by Woessner Jr. (Woessner Jr., J.F. The Determination of Hydroxyproline in Tissue and Protein Samples Containing Small Proportions of this Imino Acid. *Arch Biochem Biophys.* 1961; 93:440-447). Firstly, a solution of L-hydroxyproline (Fluka ®) (5 mg in 50 mL of 0.001 M HCl) was prepared followed by several dilutions to obtain standards of concentrations between 1-5 µg/2mL. The OHPro oxidation in each standard was initiated by adding 1 mL of chloramine T (Sigma ®) under stirring, and the solution was left to stand for 20 minutes at room temperature. After this procedure, 1 mL of perchloric acid (Nuclear ®) 3.15 M was added to each standard. The tubes were stirred and after 5 minutes at room temperature 1 mL of p-dimethylaminobenzaldehyde solution (Sigma ®) was added under stirring. The tubes were heated to 60 °C in a water bath for 20 minutes and chilled in

water for 5 minutes. The standards concentration was spectrophotometrically determined by measuring the absorbance at 557nm (Shimadzu UV - 1601PC) and the standard curve was obtained.

Then, the acid hydrolysis was made (6M HCl at 130 °C for 24 hours) of the respective samples: BC, BC-COL and (BC-COL)-HAp, followed by neutralization with 0.25 M NaOH in order not to exceed the concentration of 0.4 M NaCl, as this salt may inhibit the color development.

The OHPro content in the hydrolysed samples was determined using the protocol described above, except that after the cooling and prior to the spectrophotometric reading, the OHPro was extracted with benzene (Sigma ®). For this end, 10 ml of benzene were added to each tube, followed by stirring for 5 seconds, and the benzene layer was removed quickly. The extraction was rapidly repeated with further addition of 10mL benzene and centrifuged at low speed for 1 minute. The absorbance of the solutions was measured with a spectrophotometer at 557nm, and a standard rate of 10% hydroxyproline (1:10) was used to determine the total amount of collagen in the samples (Hollander, A.P.; Heathfield, T.F.; Webber, C.; Iwata, Y.; Bourne, R.; Rorabeck, C.; Poole, R.A. Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay. *J. Clin. Invest.*, v. 93, p.1722-1732, 1994).

4. IN VITRO ANALYSIS

The cell culture selected to perform *in vitro* studies was a primary culture of rat calvaria osteogenic cells. The following *in vitro* assays have been proposed as: 1. Staining with DAPI-phalloidin and propidium iodide, direct fluorescence analysis that assesses cell adhesion and cell spreading on the material tested to stain the ubiquitous actin cytoskeleton and to stain the nuclear DNA, 2. Cell viability analysis evaluated by MTT colorimetric assay, 3. alkaline phosphatase (ALP) analysis. The statistical analysis was performed by parametric or non-parametric tests, for independent data and comparison of two or more samples (ANOVA or Kruskal-Wallis, respectively, whether or not there are normal sampling distribution and homogeneity of variances), followed by multiple comparisons test, when applicable.

Thus, a preliminary analysis of the osteoprogenitor cells morphology from rat calvaria was performed with the following samples: BC,

BC+OGP and BC+OGP (10-14), and the composites BC-COL, (BC-COL)-HAp, (BC-COL)-HAp+OGP and (BC-COL)-HAp+OGP(10-14). Samples with the peptides were assayed at concentrations ranges of 10^{-6} and 10^{-9} M. Samples were prepared in disc shape with standard dimensions of 13mm in diameter and 0.05 mm thick to BC samples, 0.15 mm to composites (BC-COL)-HAp (n = 5) and placed into each well on 24-wells sterile acrylic plate.

4.1 CELL ISOLATION AND PRIMARY CULTURE OF OSTEOGENIC CELLS

Cells were isolated by sequential trypsin/collagenase digestion of calvarial bone from newborn (2–4 days) Wistar rats. Cells were plated on the respective membranes contained in the plates at a cell density of 2×10^4 cells/well, and grown for periods of 21 days in α Modified Minimum Essential Medium, with L-glutamine (α -MEM, Invitrogen®, USA) supplemented with 10% fetal bovine serum (Invitrogen), 7 mM β -glycerophosphate (Sigma®, USA) 50 μ g/mL gentamicin (Invitrogen®), 5mg/L ascorbic acid (Sigma®) at 37 °C in a humidified atmosphere with 5% CO₂. The culture medium was changed every 2-3 days and the cell outgrowth and morphology were assessed by phase microscopy in cultures grown on polystyrene.

4.2 CELLULAR MORPHOLOGY AND STAGES OF CELL ADHESION AND SPREADING

The cell adhesion and spreading were assessed by direct fluorescence using Alexa Fluor 488-conjugated phalloidin (Molecular Probes®, USA) for ubiquitous actin cytoskeleton staining; and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Molecular Probes®) or propidium iodide for nuclear DNA staining. The BC analyses were carried out for periods of 10 and 17 days, BC with respective peptides at concentrations above and BC-COL, for periods of 1, 3 and 7 days; and BC and (BC-COL)-HAp both containing OGP or OGP (10-14) at a concentration of 10^{-9} M.

4.3 DETERMINATION OF CELL VIABILITY AND PROPORTION OF CELLS IN CELL CYCLE

The cell viability analysis was performed by MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5- Diphenyltetrazolium) Bromide] colorimetric assay, a tetrazolium salt that is reduced by the mitochondrial proteases, and is active only in viable cells. MTT aliquots at 5mg/mL in PBS were prepared following the incubation of primary cultures with said solution at 10% in culture medium for 4 hours in a standard humidified incubator at 37°C containing 5% CO₂/95%

atmospheric air. After this period, cultures were washed with 1 mL of warm PBS. Then, 1mL of acid isopropanol solution (100mL isopropanol and 134 μ L HCl) was added in each well under stirring for 5 min to complete solubilization of the precipitate formed. Aliquots of 200 μ L were removed from the wells and transferred into 96-well plate and a spectrophotometer (570nm; μ Quanti, Biotek Instruments Inc., Winooski, VT, USA) was used for colorimetric measurement.

4.4 ALKALINE PHOSPHATASE (ALP) ACTIVITY

The ALP activity was evaluated at 14 and 21 days by thymolphthalein release from thymolphthalein monophosphate, using a commercial kit following the manufacturer's instruction (Labtest Diagnostica, Belo Horizonte, MG, Brazil). Firstly, 50 μ L thymolphthalein monophosphate were mixed with 0.5 mL of 0.3 M diethanolamine buffer, pH 10.1 and incubated for 2 min at 37 °C. To the solution it was added to 50 μ L aliquots obtained from each well for 10 min at 37 °C. For color development, 2 mL of 0.09 M Na₂CO₃ and 0.25 M NaOH were added. After 30 min, absorbance was measured using a spectrophotometer at 590 nm and alkaline phosphatase activity was determined using a thymolphthalein standard curve ranging from 0.012 to 0.4 μ mol thymolphthalein/h/mL. The data were normalized to total cell protein concentration obtained by the modified Lowry method. (Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, v. 193, p. 265-275, 1951). Total protein contents were extracted from each well with 0.1% sodium lauryl sulphate (Sigma) for 30 min and mixed 1:1 with Lowry solution (Sigma) for 20 min at room temperature. The extract was diluted in Folin and Ciocalteu's phenol reagent (Sigma) for 30 min at room temperature. Absorbance was measured at 680 nm using a spectrophotometer (Cecil CE3021, Cambridge, UK). The total protein content was calculated based on the albumin standard curve expressed as μ g/mL.

The statistical analysis was performed by parametric or non-parametric tests, for independent data and comparison of two or more samples (ANOVA or Kruskal-Wallis, respectively, whether or not there are normal sampling distribution and homogeneity of variances), followed by multiple comparisons test, when applicable.

RESULTS

1. OGP AND OGP (10-14) PEPTIDES: SYNTHESIS AND PURIFICATION

The growth factors have been synthesized by the solid-phase method (SPPS). The synthesis was carried out using 500 mg of Fmoc-Gly-
5 Wang resin (substitution level = 0.66 mmol/g) in 10 mL polypropylene syringe fitted with a porous polyethylene filter attached to a vacuum system with integrated waste collection flask.

After the coupling of the first five amino acids, the mass obtained was 720mg, which was cleaved into two equal fractions of 360mg. The first was
10 unprotected, resulting in 304mg of peptidyl-resin; the latter proceeded with the synthesis process of the OGP sequence which after deprotection resulted in 525mg of peptidyl-resin.

Each of the peptidyl-resins resulting from the synthetic process was submitted to the cleavage process by treatment with 1 ml of cleavage
15 solution (94.5 % TFA, 2.5% EDT, 0.5 % Tioanisol and 2.5 % ultra-pure water) to 100 mg peptidyl-resin at 25 °C for 2 hours.

After the cleavage procedure, the precipitation of OGP and OGP (10-14) peptides from the resin was performed with cold diethyl ether (washed 6 times) and then the extraction of the respective peptides with 10% acetic acid
20 using a 5mL polypropylene syringes systems fitted with a porous polyethylene filter attached to a vacuum system with integrated waste collection flask for each peptides extracted. The crude extract of each peptide was lyophilized and the mass value obtained for OGP and OGP (10-14) were 21.7 mg and 3.7 mg, respectively.

The peptides thus obtained were subjected to qualitative HPLC analysis; and the chromatographic profile of the crude OGP (10-14) extract showed a single peak with a relative purity of 97%, and due to the crude value (3.7 mg), it was chosen not to perform the semi-preparative scale purification of such peptide, this being regarded as pure mass (Figure 3).
25

From the results of retention time and percentage of solvent in OGP analysis, the condition of solvent concentration for the semi-preparative approaches to peptide purification was defined. The chromatograms obtained before and after the purification process are shown in Figure 4.
30

The chromatographic profile of OGP showed a single peak relative to the chromatogram analysis of the crude extract. The retention time parameter of OGP purified was maintained with respect to corresponding peak of OGP crude (data confirmed by liquid chromatography mass spectrometry, LC/ESI-MS).

5 The purification yield was calculated from the ratio relative to the crude peptide mass. The values of purification yield for the relative purities are exemplified in Table 4.

TABLE 4. PURIFICATION YIELD AND RELATIVE PURITY OF OGP(10-14) AND OGP

<u>PEPTIDE</u>				
peptide	crude peptide mass (mg)	peptide mass after purification	Purification Yield (%)	relative purity (%)
OGP (10-14)	3.7 mg	---	----	97
OGP	21.7 mg	5.2 mg	24	100

10 The value of OGP purification yield and the OGP (10-14) mass value, although low, are consistent with the purification of peptides. The results of relative purity of the peptide indicate that these methods developed were satisfactory for this purpose.

2. CHARACTERIZATION OF OGP AND OGP (10-14) PEPTIDES

15 The identity of the OGP (10-14) and OGP peptides was confirmed by respective molecular weights determination, 1523.5 and 499.5 g/mol, determined by positive ion ES-MS technique [ES m/z = 500 (M)] and [ES m/z = 1525 (M) and 763 (M+2H)⁺2] respectively.

20 The results obtained in the analyses of amino acids to determine the relative proportion of each amino acid used for the OGP and OGP (10-14) synthesis were close to theoretical values. The threonine amino acid can undergo degradation during acid hydrolysis, resulting in an increased difference between the theoretical and obtained values.

25 However, the results showed that the procedures used for the synthesis, deprotection, cleavage and purification of the sequences were achieved successfully, leading to obtain the desired sequences, as confirmed by purity, molecular mass and amino acid analysis datas of products synthesized.

3. CHARACTERIZATION OF COMPOSITES

3.1 BACTERIAL CELLULOSE-COLLAGEN (BC-COL)

30 The micrograph of the BC-COL composite (Figure 5B and 5C)

shows that collagen filled and covered throughout the BC nanostructure surface homogeneously (Figure 5A). The methodology used to obtain the BC-COL membrane allowed to develop a more compressed system, with filling the spaces between the cellulose fibrils. This is an important characteristic for a membrane to be employed as a mechanical barrier in guided bone regeneration, because favoring even more the cell occlusion function, preventing the infiltration of mesenchymal stem cells and fibroblasts, as well as the connective tissue invagination into the defective bone more effectively. Another characteristic is that the surface roughness of the membrane favors cell adhesion and spreading.

The X-ray diffractograms of BC, BC-COL membranes and type I collagen are depicted in Figure 6. The diffraction peaks at 15° and 22.5° are characteristic of cellulose. The XRD pattern for collagen resulted in a broad peak at 2θ ranging from 15° to 35° , which is a typical XRD pattern of pure collagen, showing amorphous polymer with low crystallinity (ZHANG, L.J.; FENG, X.S.; LIU, H.G.; QIAN, D.J.; ZHANG, L.; YU, X.L.; CUI, F.Z. Hydroxyapatite/collagen composite materials formation in simulated body fluid environment. *Mater. Letters.*, v. 58, p. 719-722, 2004). The typical BC crystalline phases were also observed in BC-COL membranes, but with a decrease in the intensity of the BC peaks. This decrease suggests the collagen incorporation to BC structure, conferring a more amorphous pattern to composite. Note that the BC-COL pattern does not reflect a simple mixture of BC with COL; in the XRD pattern, a change in crystal structure in the 15^{th} region was observed, with the emergence of a new peak around 16.8° .

Figure 7 shows the infrared spectra for BC samples, lyophilized pure collagen, modified bacterial cellulose and BC-COL.

The main features that characterizes the cellulose polymer are: $\sim 3450\text{ cm}^{-1}$ – OH stretching; $\sim 2900\text{ cm}^{-1}$ - alkane CH stretching and CH_2 asymmetric stretching; 2700 cm^{-1} – CH_2 symmetric stretching; 1645 cm^{-1} - OH bending; 1432 cm^{-1} - CH_2 bending; $\sim 1370\text{ cm}^{-1}$ - CH_3 bending; $\sim 1332\text{ cm}^{-1}$ - OH bending and the 1320-1030 region characterizes the C=O bending.

The band at 3500 cm^{-1} was observed in all samples, however it was presented with lower intensity for the modified BC and BC-COL samples.

This decrease in intensity might suggest that an amino acid interaction in the modified BC and BC-COL samples with the hydroxyl groups of cellulose esters has occurred, indicating probable links between BC and the amino acid, reducing the number of free OH available. It was also observed a decrease in the intensity of this band of the modified BC for the BC-COL composite, suggesting an interaction of collagen with the hydroxyl or the amino group of the amino acid from modified BC system that were still available for the cross-linking process with EDC. The band located at 2900 cm^{-1} region assigned to the CH₂ stretching was more intense for samples containing BC than for pure collagen. It was observed that in the samples of modified BC, the band intensity decreases considerably relative to the BC sample, suggesting the interaction of the amino acid with BC. In the BC-COL spectrum, there was a decrease in the band intensity relative to the BC, which suggests a greater interaction of collagen with the modified BC.

The pure collagen presents typical bands for proteins of about 1656 and 1547 cm^{-1} , relative to amide I C=O stretching and for amide II N-H bending, respectively (Figure 8).

It was observed that there is a typical band at 1645 cm^{-1} corresponding to amide I C=O stretching in the BC, modified BC and BC-COL spectra. Such band was observed with lower intensity in the BC-COL spectrum, and showed a red-shift of 11 cm^{-1} (1656 cm^{-1}), confirming the presence of collagen (Figure 8).

3.2 (BACTERIAL CELLULOSE-COLLAGEN)-HAP [(BC-COL)-HAP]

Figure 9 depicts the micrographs of the (BC-COL)-Hap composite. The figure 9B confirms the formation of the (BC-COL)-Hap composite, indicating that the membrane surface was covered with small crystals of HAp and the collagen and HAp crystals filled and covered, homogeneously, throughout the BC porous nanostructure surface. This precipitation homogeneity of HAp on the composite surface favors the improvement of cell adhesion and spreading, being an important characteristic for a membrane to be employed as a mechanical barrier in guided bone regeneration, because it has an advantage over BC-COL. Moreover, it promoted a better cell occlusion, preventing the infiltration of connective tissue into the defective bone. The (BC-

COL)-Hap system showed an increased surface roughness, promoting an improved cell adhesion and spreading of osteoblastic cells compared to BC-COL system.

The EDX mapping technique of the (BC-COL)-Hap composite showed Ca and P ions precipitation homogeneity of on the surface of respective sample (Figure 10).

The EDX data for (BC-COL)-Hap samples revealed a Ca/P molar ratio of about 1.33, these data regarding the octacalcium phosphate (OCP) precursor phase of biological apatite.

The TEM images showed nanosize HAp crystals surrounding bacterial cellulose nanofibrils. It was observed crystals of about 15 to 100nm (Figure 11). These nanometric structures of HAp crystals and BC fibrils promote an improved cell adhesion and spreading. Moreover, as these crystals are nanometric, they are more easily dissolved by the body and thus there is an availability of Ca^{2+} and PO_4^{3-} faster in the biomaterial/tissue interface, promoting a cell proliferation and differentiation in periods earlier than with micrometer HAp crystals with the same chemical composition of such HAp crystals of the (BC-COL)-Hap composite.

Figure 12 shows the XRD pattern for the (BC-COL)-Hap composite, with similar crystallinity patterns for the HAp of bone tissue. The significant peaks observed for the HAp crystals in the samples were in 26° , 29° , 32° , 40° and 46° .

However, the broadening of diffraction peaks related to HAp observed in these samples suggests that the crystals precipitated were small and with low crystallinity. It is suggested that the inorganic phase of the composite (BC - COL)- HAp mineralized HAp was a low crystalline apatite similar to bone tissue (DANILCHENKO, S.N.; MOSEKE, C.; SUKHODUB, L.F.; SULKIO-CLEFF, B. X-ray diffraction studies of bone apatite under acid demineralization. *Cryst. Res. Technol.*, v. 39, p.71–77, 2004). The typical crystalline phase of the BC peak at 22.5° was also observed in the (BC-COL)-HAp composite at lower intensity than the XRD pattern to BC, and the respective peaks at 15° and 16.8° decrease significantly due the changes in the pattern of crystallinity of the composite. Such decrease in the intensity of the

peaks was quite evident, as it was related to the collagen incorporation and deposition of HAp on the BC nanofibers.

Figure 13 shows the FT-IR (BC-COL)-Hap spectra. The typical infrared bands of phosphate (PO_4^{3-}) associated with the apatite structure which appeared around 1093, 1020, 570-600 cm^{-1} are assigned to the stretching of the (PO_4^{3-}) ions (WAN, Y. Z.; HUANG, Y.; YUAN, C. D.; RAMAN, S.; ZHU, Y.; JIANG, H. J.; HE, F.; GAO, C. Biomimetic synthesis of hydroxyapatite/bacterial cellulose nanocomposites for biomedical applications. *Mater. Sci. Eng. C.*, v. 27, p. 855–864, 2007; CROMME, P.; ZOLLFRANK, C.; MULLER, L.; MULLER, F. A.; GREIL, P. Biomimetic mineralization of apatites on Ca^{2+} activated cellulose templates. *Mater. Sci. Eng. C.*, v., 27, p. p.1-7, 2007). Such bands had a broad aspect, suggesting the presence of HAp with low crystallinity (YUNOKI, S.; MARUKAWA, E.; IKOMA, T.; SOTOME, S.; FAN, H.; ZHANG, X.; SHINOMIYA, K.; TANAKA, J. Effect of collagen fibril formation on bioresorbability of hydroxyapatite/collagen composites. *J. Mater. Sci. Mater. Med.*, v. 18, p. 2179-2183, 2007). These results corroborate the XRD data. The weak bands located at 1428 and 870 cm^{-1} corresponding to the CO_3^{2-} ions stretching were also observed in the (BC-COL)-Hap spectrum.

With XRD and FT-IR data, it was possible to confirm that the methodologies employed to perform the HAp incorporation into composites provided a precipitation of HAp crystals with similar structures to biological apatite.

The band at 3500 cm^{-1} observed in the composites (BC-COL)-Hap spectrum appeared with greater intensity than in the BC-COL composite, suggesting that this increase may be related to the OH group from hydroxyapatite precipitated on their respective systems or higher water content in the composite.

The band peaked at 2900 cm^{-1} assigned to CH_2 stretching was less intense to (BC-COL)-Hap sample as compared to BC-COL sample, suggesting that the CH_2 stretching was lower due to the interaction of hydroxyapatite crystals in the sample. With the incorporation of the HAp to BC-COL system it became difficult to assign bands to the collagen in the IR spectra for the (BC-COL)-Hap composites.

However, the hydroxyproline analysis clarified the amount of collagen present in each composite developed. The proportions of the collagen from BC-COL and (BC-COL)-HAp composites are shown in Table 5.

TABLE 5. RELATIVE PERCENTAGE OF HYDROXYPROLINE AND COLLAGEN IN SAMPLES

5

ANALYZED

Samples	Weight (mg)	OHPro (%)	Collagen (%)
BC-COL	23.8	0.37	3.7
(BC-COL)-HAp	17.3	0.07	0.7

4. IN VITRO ANALYSIS

4.1 CELLULAR MORPHOLOGY

The epifluorescence analysis of osteogenic cultures revealed that the BC and modified BC membranes promoted the cell adhesion and proliferation. On day 10, the cells were not spread evenly over BC, the majority was concentrated on cell clusters, possibly due to intrinsic characteristics of these membranes (Figure 14A). In other groups, cells were spread throughout the surface, forming multilayered areas (Figure 14B and C). On day 17, it was observed an increase in cell population over BC (Figure 15A). There was no significant differences between the cultures grown over other membranes in relation to standard shown at 10 days (Figure 15B, C, D, E and F).

Figure 16 shows the cell adhesion and proliferation at the time periods of 1, 3 and 7 days to (BC-COL)-HAp with or without growth factor. In the respective samples, only the sample of day 7 (BC-COL)-HAp_OGP(10-14) showed cells distributed throughout the surface areas forming multilayers (Fig. 16 - I).

4.2 CELL PROLIFERATION

At 14 and 21 days, cell proliferation was quantitatively determined by the by MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5- Diphenyltetrazolium) Bromide] colorimetric assay. Cell proliferation was measured by absorbance values obtained and the mean and standard deviation for each group used to construct the graph of cell proliferation (Figure 16). The original values were tested using nonparametric Kruskal-Wallis statistical test for independent data (Table 6).

The Kruskal-Wallis test showed significant difference, at 0.05 significance level, for the absorbance values obtained at 14 and 21 days.

Cultures grown over BC, BC_OGP 10^{-9} M and BC_OGP(10-14) 10^{-9} M showed higher absorbance values in both 14 and 21 days, while the lowest values were obtained in cultures grown on (BC-COL)-HAp at 14 days, and (BC-COL)-HAp and (BC-COL)-HAp_OGP (10-14) 10^{-9} M at 21 days.

- 5 It was noted a reduction in absorbance values between 14 and 21 days, regardless of the membrane (Mann-Whitney test, $p < 0.05$). This decrease in cell proliferation may be related to the progression of osteoblast differentiation.

10 **TABLE 6. COMPARATIVE ANALYSIS (FISCHER TEST BASED ON RANK) OF ABSORBANCE VALUES OBTAINED IN THE CELL PROLIFERATION ASSAY AT 21 AND 14 DAYS (KRUSKAL-WALLIS, $P < 0.01$).**

Comparisons (post tests)	MTT 14 days	MTT 21 days
BC x BC_OGP 10^{-9} M	ns	ns
BC x BC_OGP (10-14) 10^{-9} M	ns	ns
BC x (BC-COL)-HAp	1%	1%
BC x (BC-COL)-HAp_OGP 10^{-9} M	ns	ns
BC x (BC-COL)-HAp_OGP(10-14) 10^{-9} M	5%	1%
BC_OGP 10^{-9} M x BC_OGP(10-14) 10^{-9} M	ns	ns
BC_OGP 10^{-9} M x (BC-COL)-HAp	1%	1%
BC_OGP 10^{-9} M x (BC-COL)-HAp_OGP 10^{-9} M	ns	ns
BC_OGP 10^{-9} M x (BC-COL)-HAp_OGP(10-14) 10^{-9} M	ns	1%
BC_OGP(10-14) 10^{-9} M x (BC-COL)-HAp	5%	1%
BC_OGP(10-14) 10^{-9} M x (BC-COL)-HAp_OGP 10^{-9} M	ns	ns
BC_OGP(10-14) 10^{-9} M x (BC-COL)-HAp_OGP(10-14) 10^{-9} M	ns	1%
(BC-COL)-HAp x (BC-COL)-HAp_OGP 10^{-9} M	ns	ns
(BC-COL)-HAp x (BC-COL)-HAp_OGP(10-14) 10^{-9} M	ns	ns
(BC-COL)-HAp_OGP 10^{-9} M x (BC-COL)-HAp_OGP(10-14) 10^{-9} M	ns	ns

4.3 ALKALINE PHOSPHATASE (ALP) ACTIVITY

- 15 The ALP activity was measured by thymolphthalein release from the thymolphthalein monophosphate hydrolysis. The ALP is an enzyme present in the membrane of mature osteoblasts, whose function is to hydrolyze phosphate groups present in organic molecules, thus releasing phosphate ions, which are necessary for the process of mineralization.

During a process of guided tissue regeneration, the cells adhere to the

membrane, proliferate and differentiate, which stop cycling before starting the differentiation process. Therefore, it was expected that groups with low values for MTT (low proliferative rate) presented the highest values of alkaline phosphatase, thus, higher values of enzyme activity favors the mineralization process.

5 Data from this analysis were submitted to analysis of variance (ANOVA) with a single factor (treatment x membrane), followed by Duncan's test when appropriate.

Figure 17 shows the ALP activity in the BC, BC_OGP 10^{-9} M, BC_OGP (10-14) 10^{-9} M, (BC-COL)-HAp, (BC-COL)-HAp_OGP 10^{-9} M and (BC-COL)-HAp_OGP(10-14) 10^{-9} M membranes. The membranes promoted the ALP activity as follows: (BC-COL)-HAp_OGP 10^{-9} M > (BC-COL)-HAp_OGP(10-14) 10^{-9} M > (BC-COL)-HAp = BC_OGP (10-14) 10^{-9} M = BC = BC_OGP 10^{-9} M (F=3.8509; p=0.0151).

15 The results obtained by ALP activity assay suggested that the membranes of (BC-COL)-HAp_OGP 10^{-9} M and (BC-COL)-HAp_OGP (10-14) 10^{-9} M favored the mineralization process.

The low levels of ALP activity in the other membranes may be related to the delay observed in cell adhesion and proliferation. Thus, the ALP activity peak of cultures grown on BC, BC_OGP 10^{-9} M, BC_OGP (10-14) 10^{-9} M and (BC-COL)-HAp probably occurs in periods exceeding 21 days.

CONCLUSION

25 The composites showed a homogenous distribution of collagen and HAp crystals on the matrix of BC or BC-COL composites (in the case of crystals) with a homogeneous nanostructure via *in situ* synthesis at room temperature. The composite (BC -COL)-HAp showed similar characteristics to natural bone, such as phase composition, crystal size and crystallinity.

The composites synthesized by biomimetic route promoted excellent cell proliferation and, therefore, the BC-COL and (BC-COL)-HAp composites of the present invention are useful biomaterials for tissue repair, particularly bone.

EXAMPLE 2

COMPARATIVE TESTS ON THE ESTERIFICATION METHODOLOGY

The present inventors conducted a study comparing two methods

of activation: i) DIC/NMI used in the present invention, where activation occurs *in situ*, and ii) the traditional DIPCDI/DMAP normally used in the prior art, with pre-activation, in the absence of catalyst (formation of symmetrical anhydride) and subsequent reaction by adding the catalyst.

5 In both methods were employed the same conditions, namely:

Fmoc-glycine: 0.8 mmols

Reaction time: 2 horas

Reaction volume: 8 mL

Amount of reagents: 1 equivalent relative to Fmoc-Gly.

10 After the reaction and washing period, the two materials were analyzed for incorporation of Fmoc-Gly. Then, the spectrophotometric analysis was employed by assessing the absorption intensity at 290 nm after treatment of samples with piperidine.

The results are given below.

Method	DIPCDI/DMAP	CDI/NMI
Fmoc-Gly (mmol/g)	0.52×10^{-3}	4.0×10^{-3}

15 The results illustrate the superior performance obtained with the CDI/NMI activation, as used by the present invention.

As commonly understood by one of ordinary skill in the art, it is recognized that various modifications and variations of the present invention are possible in light of the teachings above without departing from the spirit and the
20 scope of the invention, as defined by the appended claims.

CLAIMS

1. A method for obtaining reabsorbable composites based upon bacterial cellulose and collagen, wherein the method comprises:

a) obtaining of bacterial cellulose;

5 b) esterification of an amino acid or an amino acid derivative to the membrane of bacterial cellulose, using as activating agents the carbonyldiimidazole (CDI) and N-methylimidazole (NMI); and

c) incorporation of collagen, with the proviso that when one amino acid derivative is used, such incorporation is preceded by deprotection of the amine group from the amino acid derivative incorporated into the membrane.

2. The method of claim 1, wherein the bacterial cellulose is obtained by culturing the *Acetobacter xylinum* bacteria in static culture medium.

3. The method of claim 1, wherein the amino acid or amino acid derivative in step (b) is aliphatic and nonpolar.

15 4. The method of claim 3, wherein the amino acid is glycine (Gly) and amino acid derivative is 9-Fluorenylmethyloxycarbonyl-glycine (Fmoc-Gly).

5. The method of any one of claims 1–4, wherein the esterification in step (b) is made with an amino acid derivative.

20 6. The method of claim 1, wherein the said step (c) of the present invention is performed so that after the deprotecting of the amino group, when necessary, a covalent bond between the amino acid or amino acid derivative and the collagen that will be inserted are performed using an aqueous solution containing collagen and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.

25 7. The method of claim 6, wherein the temperature during the linking between the amino acid and the collagen in the step (c) is about 0 °C to 10 °C.

8. The method of claim 1, further comprising the additional hydroxyapatite (HAp) precipitation and incorporation step following the step (c).

30 9. The method of claim 8, wherein the said additional hydroxyapatite precipitation and incorporation step is accomplished by:

- incorporating hydroxyapatite by methods known in the art; and

- immersing the composite cellulose-aa-collagen first in a solution containing calcium and then in solutions containing phosphate.

10. The method of claim 9 wherein all cycles of incubation for HAp incorporation are performed preferably at room temperature for 24 hours.

5 11. The method of any one of claims 1–10, further comprising a subsequent incorporation of peptides that induce the cell proliferation and/or differentiation.

12. The method of claim 11, wherein the HAp incorporation is carried out maintaining the composites in solution containing said peptides.

10 13. The method of claim 11, wherein the peptides concentration in solution is in the range of 10^{-6} to 10^{-9} M.

14. The method of any one of claims 11–13, wherein the said peptides are incorporated into the cellulose matrix through adsorption.

15 15. The method of any one of claims 11–14, wherein the said peptides are selected from factors or modulators of growth and antimicrobial.

16. The method of claim 15, wherein said peptides are OGP (osteogenic growth peptide) or OGP (10–14) (osteogenic growth peptide (10–14)).

20 17. The method of any one of claims 1–16, wherein the composites obtained of the present invention may be subsequently dried at 37 °C for the manufacture of membranes for tissue repairing.

18. The method of any one of claims 1–16, wherein the composites obtained of the present invention may be subsequently freeze-dried for making scaffolds.

25 19. A composite obtained from the method of any one of claims 1 to 16.

20. A membrane obtained from the method of claim 17.

21. A scaffold obtained from the method of claim 17.

22. Use of a composite of claim 19, wherein the said composite is used for tissue repairing.

30 23. Use according to the claim 22, wherein the said tissue is a bone or connective tissue.

24. Use of a membrane of claim 20, wherein the said membrane is used for tissue repairing.

25. Use according to the claim 24, wherein the said tissue is a bone or connective tissue.

26. Use of a scaffold of claim 21, wherein the said scaffold is used for tissue repairing.

5 27. Use according to the claim 26, wherein the said tissue is a bone or connective tissue.

28. A composite comprising, respectively, bacterial cellulose, collagen and hydroxyapatite.

10 29. Use of a composite of claim 28, wherein the said composite is used for bone tissue repairing.

H-R-L-K-R-Q-G-R-T-L-Y-G-F-G-G-OH

OGP

H-Y-G-F-G-G-OH

OGP (10-14)

Primary structure of OGP e OGP (10-14)

FIGURE 1

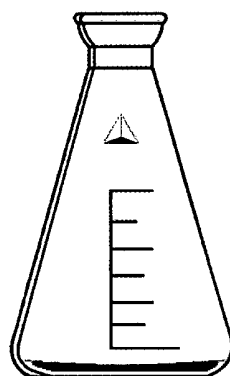
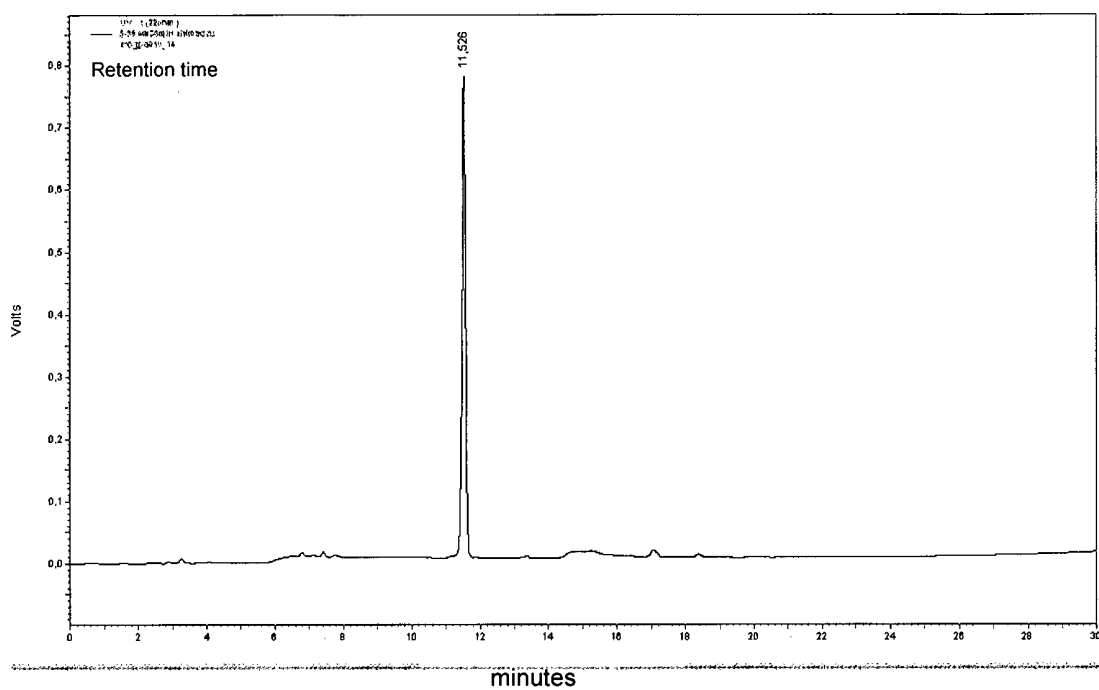


FIGURE 2



Analytical-scale chromatogram of the OGP (10-14).

FIGURE 3

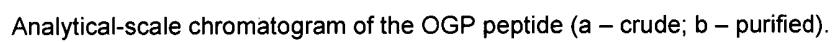
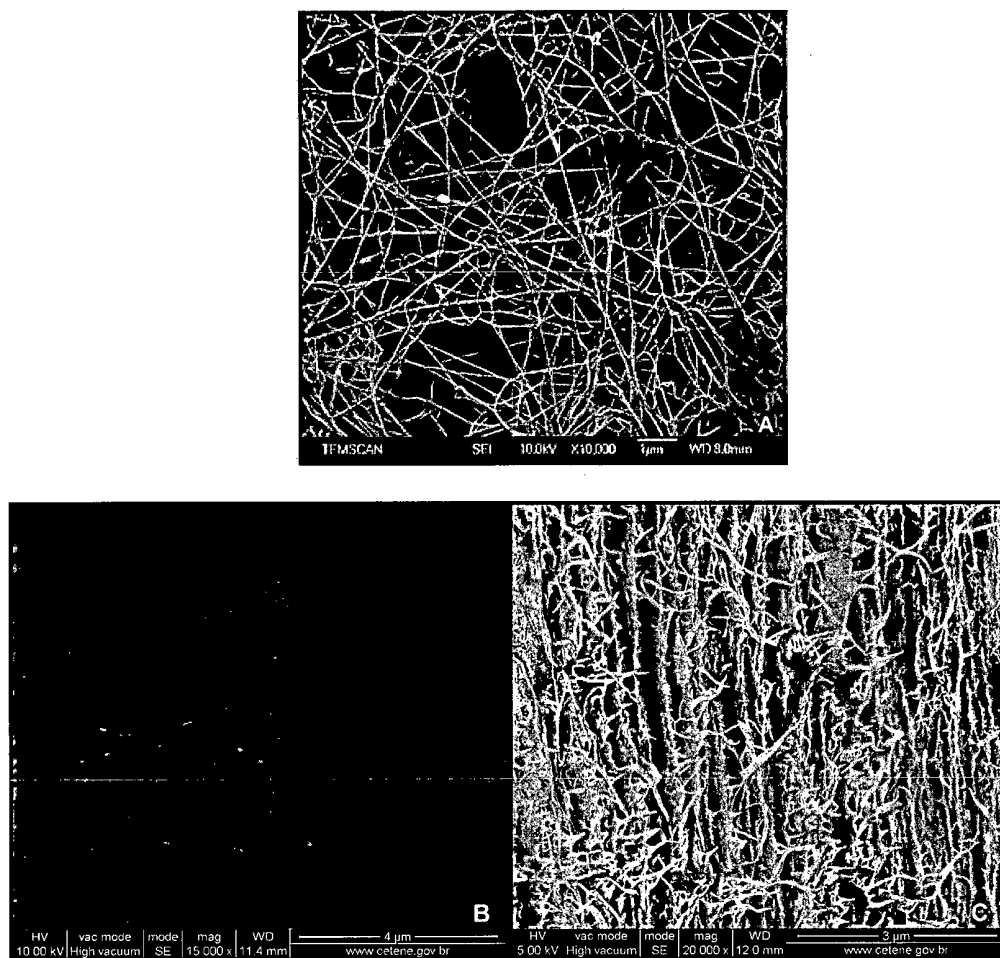
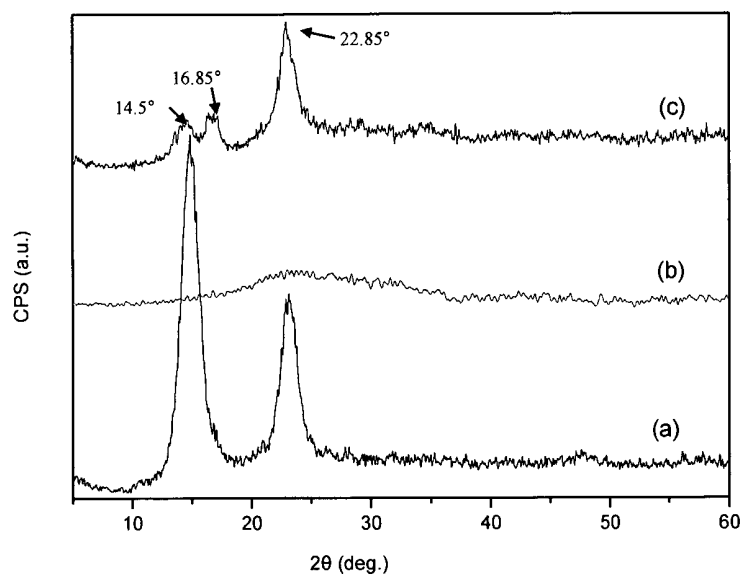


FIGURE 4



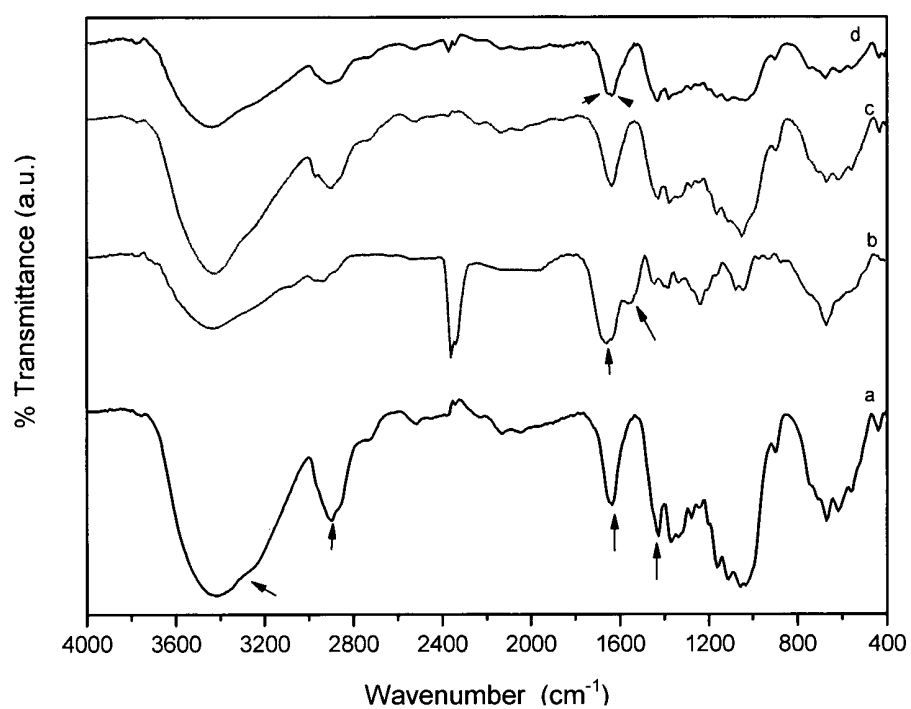
SEM micrographs : A = CB membrane; B = CB-COL membrane; C = cross section of the CB-COL membrane.

FIGURE 5



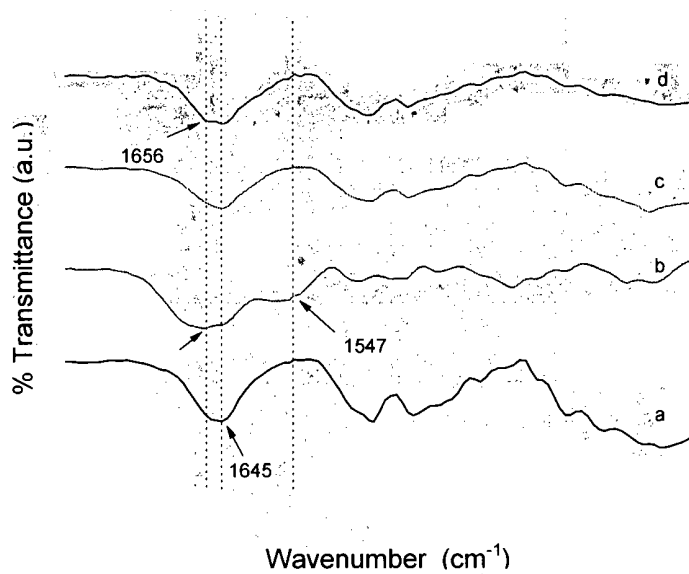
XRD diffraction patterns of the (a) CB, (b) COL, (c) CB-COL.

FIGURE 6



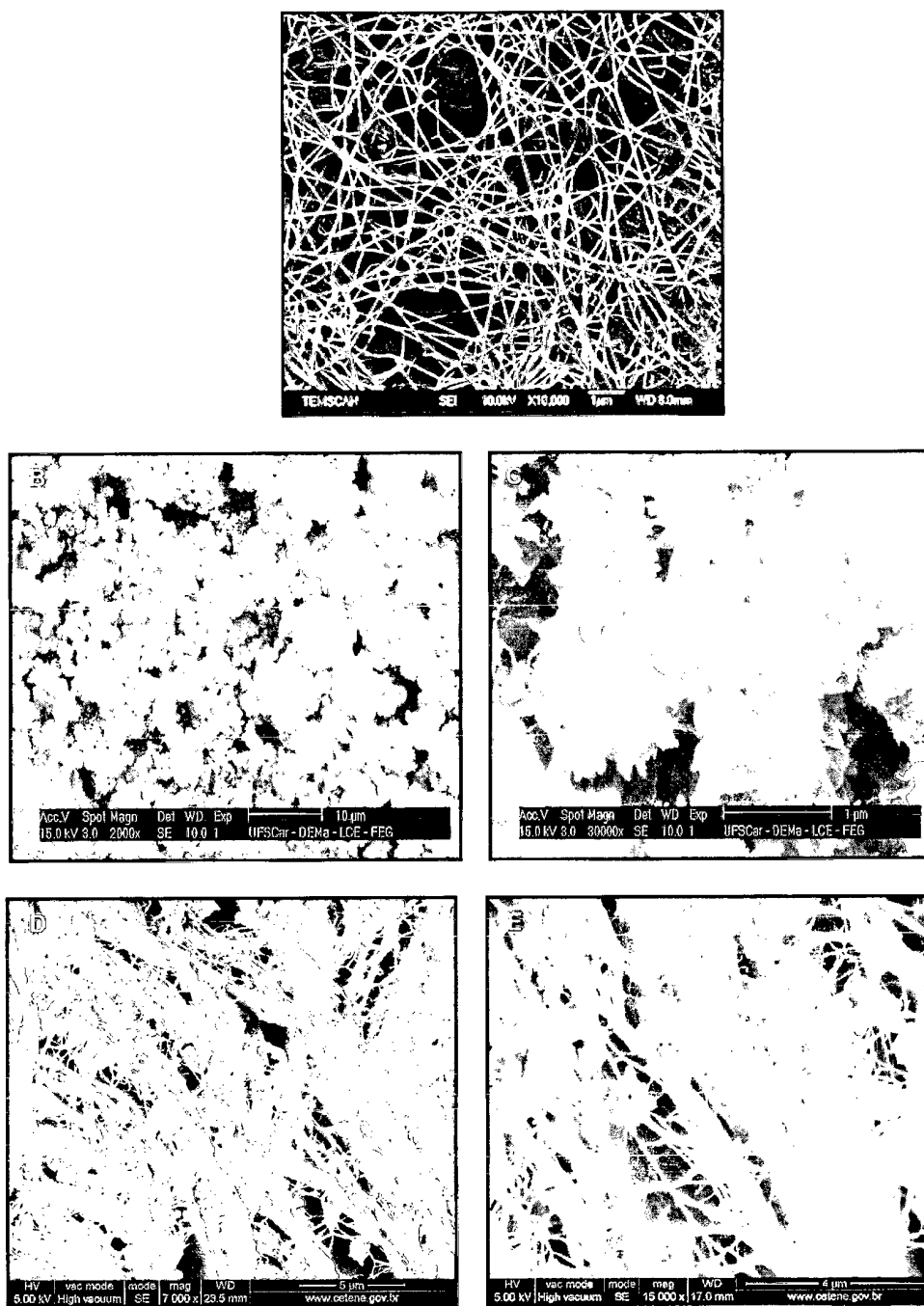
Vibrational spectrum in the infrared region from samples: BC (a), pure collagen (b), BC-glycine (c) and BC-COL composite (d).

FIGURE 7



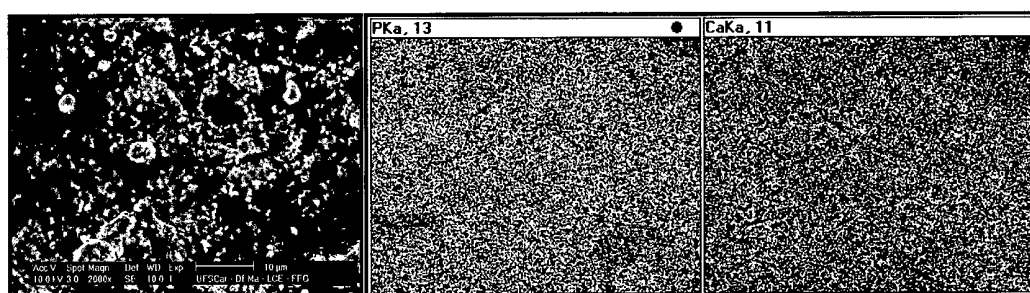
Vibrational spectrum in the infrared region between 1000 and 1850 cm⁻¹ obtained from samples: BC (a), pure collagen (b), BC-glycine (c) and BC-COL composite (d).

FIGURE 8



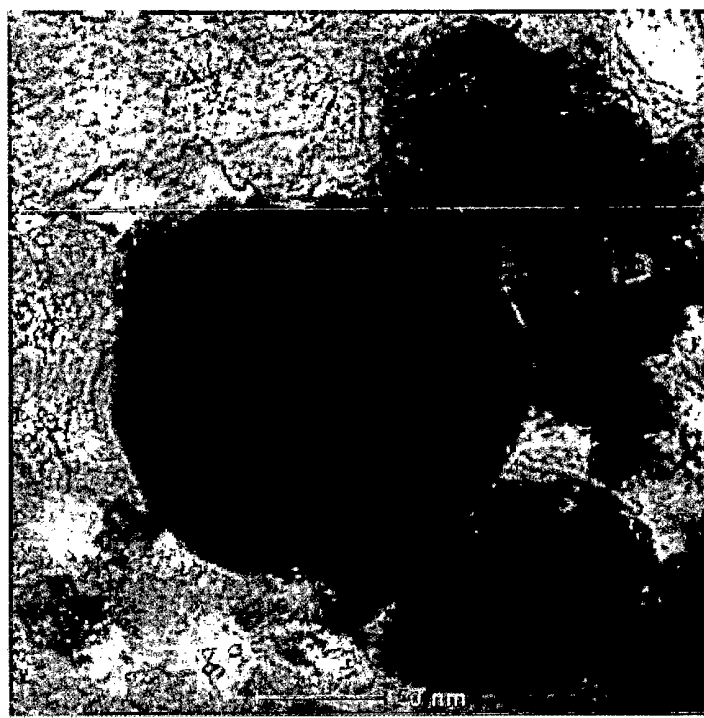
SEM micrographs: A - BC membrane, B and C - nanocomposite (BC-COL)-Hap with their respective magnifications, 2.000x and 30.000x, D and E - nanocomposite (BC-COL)-Hap in cross section with their respective magnifications 7.000x and 15.000x.

FIGURE 9



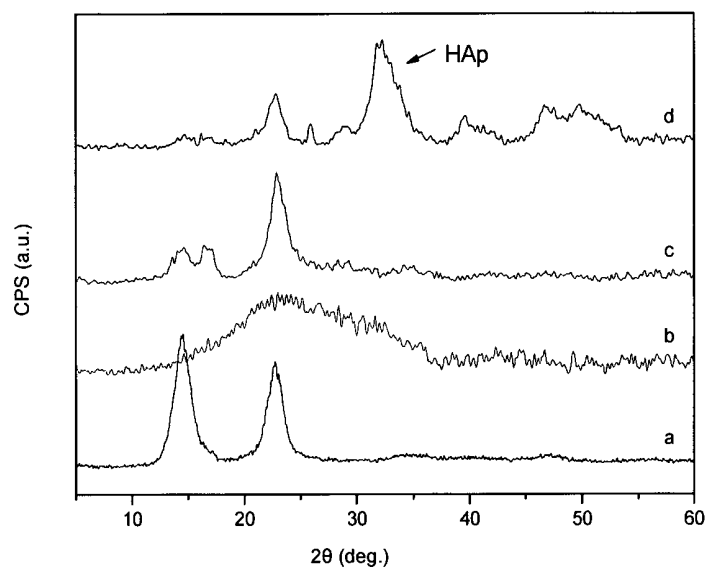
P and Ca ions mapping of the (BC-COL)-HAP composite

FIGURE 10



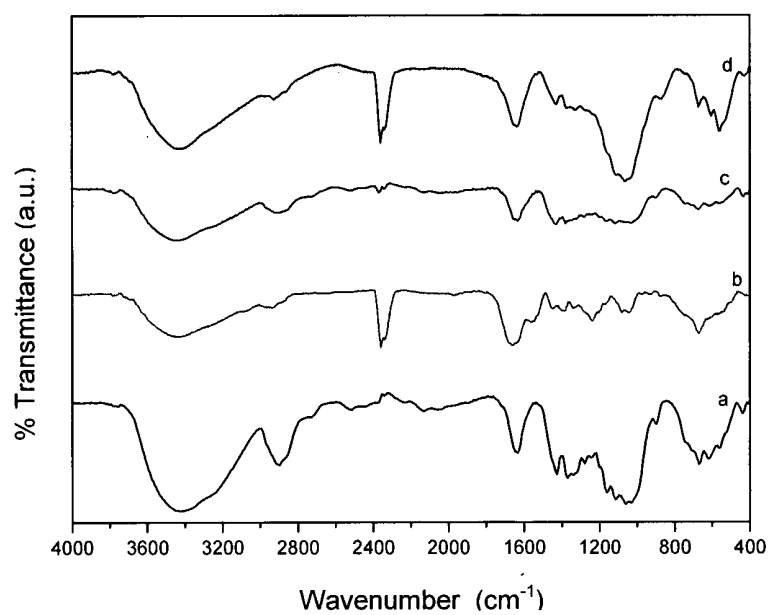
TEM image of the (BC - COL)-HAp composite.

FIGURE 11



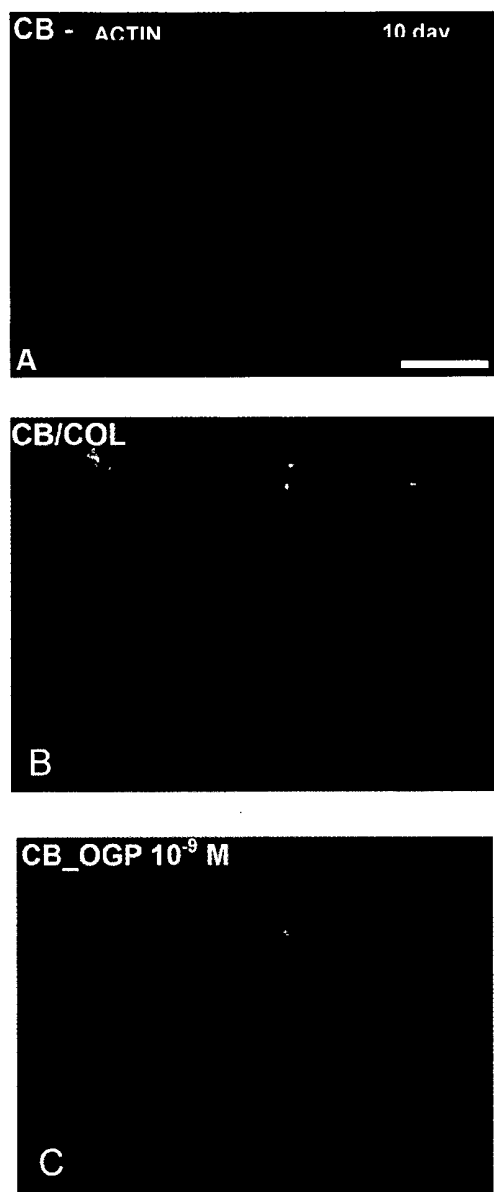
XRD diffraction patterns of (a) BC, (b) COL, (c) BC-COL and (d) (BC-COL)-HAp.

FIGURE 12



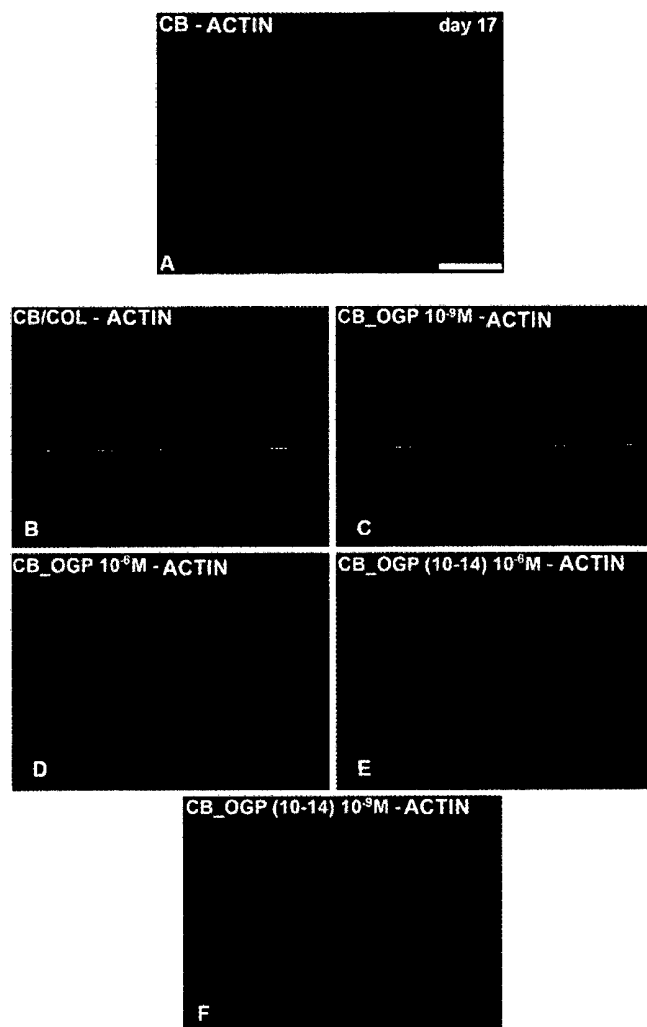
Vibrational spectrum in the infrared region obtained from samples: (a) BC, (b) COL, (c) BC - COL, (d) (BC-COL)-HAp.

FIGURE 13



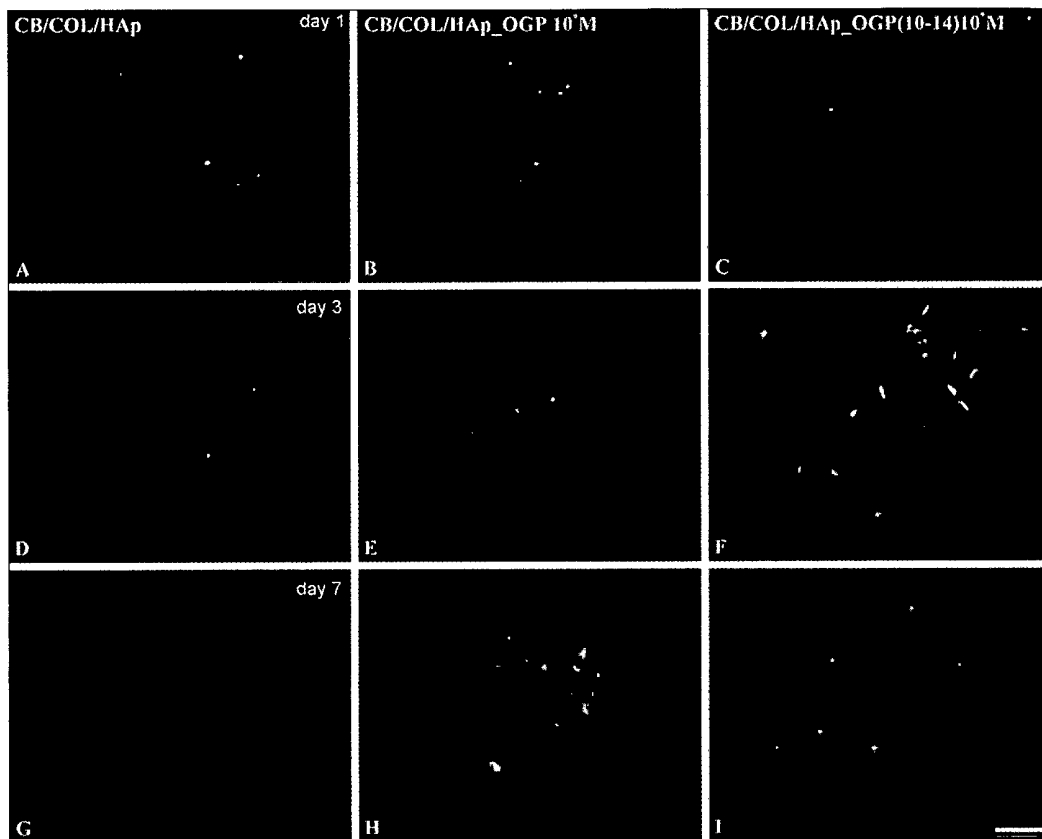
Epifluorescence of the osteoblastic cells cultured on BC (A), BC-COL (B) and BC_OGP 10⁻⁹ M (C). The green fluorescence indicates actin cytoskeleton staining. Scale Bar: 100 μ m for A-C.

FIGURE 14



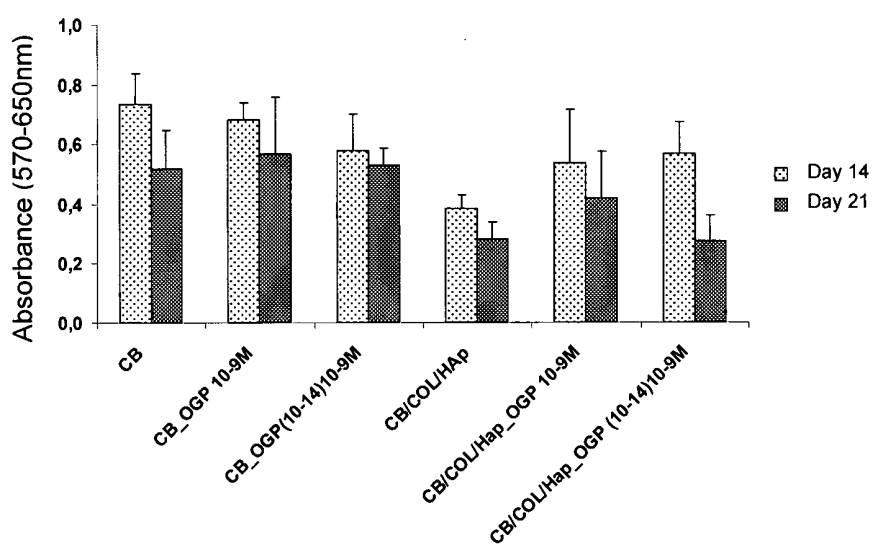
Epifluorescence of the osteoblastic cells cultured on BC (A), BC/COL (B), BC_OGP 10^{-9} M (C), BC_OGP 10^{-6} M (D), BC_OGP (10- 14) 10^{-6} M (E) and BC_OGP (10-14) 10^{-9} M (F). The green fluorescence indicates actin cytoskeleton staining. Scale Bar: 100 μ m for A-C.

FIGURE 15



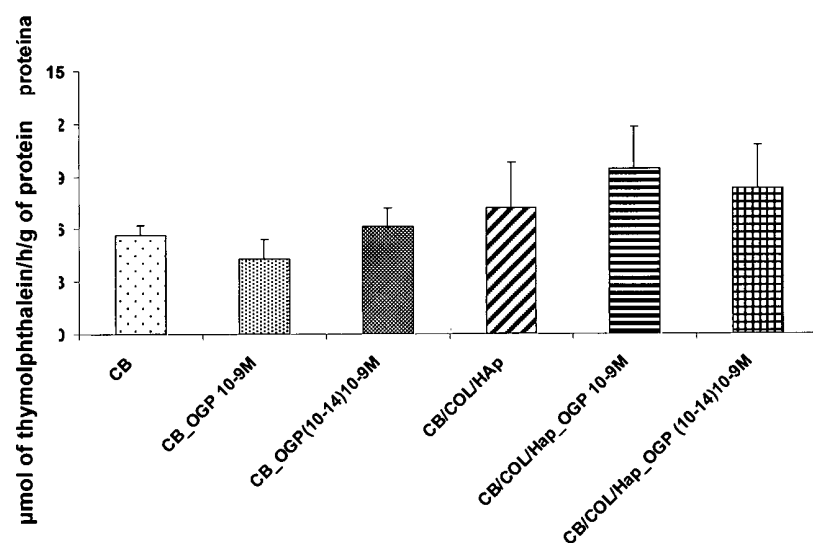
Epifluorescence of the osteoblastic cells cultured on (BC-COL)-HAp (A), (D) and (G), (BC-COL)-HAp_OGP 10^{-9} M (B), (E) and (H) and (BC-COL)-HAp_OGP (10-14) 10^{-9} M (C), (F) and (I). Green fluorescence indicates actin cytoskeleton staining and red fluorescence indicates nuclear DNA staining. Scale Bar: 100 μ m for A-C.

FIGURE 16



Cell proliferation of osteoblastic cells cultured on BC and (BC-COL)-Hap membranes associated or not with growth factors, assessed at 14 and 21 days.

FIGURE 17



Alkaline phosphatase (ALP) activity of osteoblastic cells cultured on BC and (BC-COL)-Hap membranes associated or not with growth factors, assessed at 21 days.

FIGURE 18

INTERNATIONAL SEARCH REPORT

International application N°

PCT/BR2011/000169

A. CLASSIFICATION OF SUBJECT MATTER

A61L27/26, (2006.01), A61L27/46, (2006.01), A61L27/58, (2006.01), A61L27/54, (2006.01), A61L24/08, (2006.01), A61L24/10, (2006.01), A61K35/32, (2006.01), A61K35/74, (2006.01), A61K35/36 (2006.01), A61K38/18, (2006.01), A61K38/39, (2006.01), A61K47/48, (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61L, A61K 35, A61K 38/18, A61K38/39, A61K47/48

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Portal Capes (database Banco de Teses), Plataforma Lattes CNPq

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Dialog (Database WPI b351)Epoque (database Epoque and NPL), NCBI (database pubmed)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claims N°
X	Saska et al., 2009 Nanocompósito de [celulose bacteriana-colágeno] – hidroxiapatita associado a fatores de crescimento para regeneração óssea. Revista Brasileira de Cirurgia e Traumatologia Buco-Maxilo-Facial, 6(1):86	1-29
X	EP 2070557 A1 (XYLOS CORP [US]) 17 June 2009 (2009-06-17) claims 1,7, 10 and 12	19-29
X	CN 101509025 A (UNIV WUHAN SCIENCE ENG [CN]) 19 August 2009 (2009-08-19) abstract	1-29

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

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"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

08 July 2011

Date of mailing of the international search report

220711

Name and mailing address of the ISA/BR



INSTITUTO NACIONAL DA
PROPRIEDADE INDUSTRIAL
Rua Mayrink Veiga nº 9, 18º andar
cep: 20090-050, Centro - Rio de Janeiro/RJ

Facsimile N°:

+55 21 3039-3663

Authorized officer

Karla Consort Ribeiro

Telephone N°:

+55 21 3039-3493/3742

INTERNATIONAL SEARCH REPORT

International application N°

PCT/BR2011/000169

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claims N°
A	RU 94151 U1 20 May 2010 (2010-05-20) abstract	1-29
A	Honglin et al., 2008 Preparation and characterization of a novel col/bc composite for potential tissue engineering scaffolds. Materials Chemistry and Physics 110:193-196	1-29
A	Wiegand et al., 2006 Protease and ROS activities influenced by a composite of bacterial cellulose and collagen type I in vitro. Cellulose 13:689-696	1-29
A	Czaja et al., 2006 Microbial cellulose-the natural power to heal wounds. Biomaterials 27:145-151 -----	1-29

Information on patent family members

PCT/BR2011/000169

Form PCT/ISA/210 (patent family annex) (July 2009)