Bacterial Cellulose Grafted with Acidic Groups for Biomedical Applications

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The paper focuses on the synthesis and evaluation of biomineralization potential of composite materials based on bacterial cellulose and synthetic acrylic polymers with negative groups attached. The composite materials were obtained by grafting the monomers carrying negative groups onto the surface of bacterial cellulose. These grafted membranes were then subject to mineralization assay according to T. Kokubo protocol. Cytototoxicity test have shown that this kind of composites did not show a significant in vitro cytotoxicity and could be used for developing biomedical applications.

Keywords: bacterial cellulose, acrylic monomers, biomineralization, hydroxyapatite

The development of innovative composite materials for bone tissue engineering is a very interesting and challenge task. Various polymeric composite scaffolds have been used as substitutes for the extracellular matrix, which is rapidly becoming the most challenging experimental approach for regenerating the native structural and functional properties of living tissue [1-6]. These materials may consist of natural macromolecules and synthetic polymers, providing an adhesive substrate that can serves as a 3D physical support matrix for tissue regeneration. Natural bone is a composite in which inorganic apatite nanocrystals are deposited on organic collagen fibers woven into a three-dimensional structure. An interesting task is to mimic bone structure in the design of novel bone-repairing materials [4-10].

Bone defects are currently treated with natural bone grafts (xenografts, allografts and autografts). A lot of problems were associated with the use of xenografts (especially since mad cow disease) or allografts (possibility of viral disease transfer, difficulty in reshaping the donor bone to fit defect) and autografts (limited availability, post-operative pains, donor site morbidity). All of these aspects have led to extensive research in the field of synthetic materials usable as bone substitutes, in order to obtain suitable biomaterials [1-2].

The obtaining of new materials for skeletal recovery represents a major challenge in the field of biomaterials. These materials should cover a wide spectrum of performance comprising structural and bioactive functions. A direct bone-bonding could be obtained through nucleation and growth of a calcium phosphate layer at the surface of the biomaterial. Many research teams tried to obtain hydroxyapatite (HA)-polymer composite materials. These biomaterials would improve both the mechanical properties of orthopaedic implants and their tolerance by the neighbouring tissues and interface reactions with the healthy bone tissue.

Cellulose is the most abundant biopolymer on Earth recognized as the major component of plant biomass, but also of microbial extracellular matrix [11-13].

Cellulose has traditionally been sourced from plants. However, refining of plant cellulose typically involves harsh, aggressive processing to remove noncellulose materials such as lignin and hemi-cellulose. Fortunately, an alternative source of cellulose where no chemical or mechanical refining is necessary is available. Bacterial cellulose (BC) has been developed as an alternative to plant cellulose. Due to its high water-holding capacity, high crystallinity, high tensile strength and fine web-like network structure, which means that it can be formed into any size or shape, BC could be used as a promising biomaterial [14-18].

Bacterial cellulose (BC) belongs to the products of primary metabolism and it has a protective role whereas plant cellulose plays a structural role. BC is mainly produced by *Acetobacter, Rhizobium, Agrobacterium and Sarcina*. *Acetobacter xylinum* is the only species known to be capable of producing cellulose in commercial quantities. In terms of chemical structure, bacterial cellulose is identical to that produced by plants. However, bacterial cellulose membranes possess excellent mechanical strength and high surface area when compared to plant derived cellulose due to the highly crystalline structure and reduced fiber diameter. These properties make it an interesting biomaterial for applications as nutritional component, artificial skin, composite reinforcement, nerve regeneration etc [14-19].

The use of polymeric composite materials for bone repair allows the synthesis of numerous biomaterials with specific structure and properties. The use of composite materials based on a natural polymer such as bacterial cellulose and synthetic polymers could be a very challenging problem in osseous applications. To form apatite deposits on the implant, polymeric constructs should contain negative chemical groups, in order to mimic the activity of bone proteins responsible for mineralization.

With this research we have tried to obtain composite biomaterials, which after implantation would help the local formation of mineral osseous phase (HA); these materials would adhere very well to the osseous fragments near them, which also contain HA. The advantage of this type of link between implant and bone consist in the direct welding of the two types of materials through the same material, HA. The formation of HA will lead to a composite material that *in vivo* will attract cells that will start to reconstruct the bone, and maybe, in the case of a biodegradable implant, to gradually replace the implant with the new bone.

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Therefore, this paper focuses on the obtaining and evaluation of biomineralization potential of composite materials based on bacterial cellulose and synthetic acrylic polymers with negative groups attached.

Experimental part

Materials and methods

Bacterial cellulose membranes were kindly provided by National Institute for Chemical Pharmaceutical Research and Development (ICCF Bucharest, Romania). The microorganism used in all the experiments for obtaining BC was *Acetobacter xylinium DSMZ* (ICCF 398).

Monomers 2-hydroxyethyl methacrylate (HEMA, fig. 1), itaconic acid (IA, fig. 2) and 2-acrylamido-2-methylpropane sulphonic acid (AMPSA, fig. 3) and all other reagents were provided by Sigma-Aldrich.

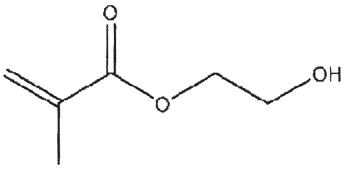

Fig.1. 2-hydroxyethyl methacrylate monomer chemical structure

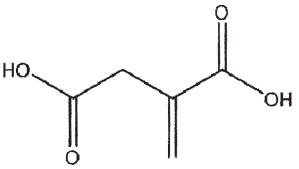

Fig.2. Itaconic acid monomer chemical structure

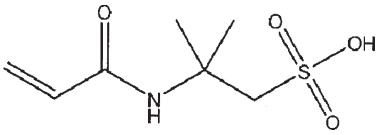

Fig.3. 2-acrylamido-2-methylpropane sulphonic acid monomer chemical structure

Hydrated (99% water) BC membranes (5 mm thick) were carefully dried at 30°C for 48 h and used for composites preparation.

The composite materials were obtained by grafting the monomers carrying negative groups onto the surface of bacterial cellulose. This method was proposed by Y. Ogiwara in 1968 [19] for fibrous plant cellulose and we have adapted it for BC membranes. Briefly the cellulose membranes were weighed and introduced in an acid solution of ammonium cerium nitrate (reaction catalyst), with nitrogen bubbling for 30 min. The monomers mixture (IA, HEMA, AMPSA, HEMA-IA and HEMA-AMPSA) and sulphuric acid 0.1N were added, followed by nitrogen bubbling for 30 min. The reaction temperature was 45 °C, in inert atmosphere, for 24 h. At the end of the reaction, the resulted materials were washed with demineralised water and extracted in order to completely eliminate the residual monomers. The reaction recipes are shown in table 1.

These grafted membranes were then subject to mineralization assay according to T. Kokubo protocol [20-23]. Briefly three samples of each specimen composition were incubated in synthetic body fluid (SBF1x) at *p*H=7.4, adjusted with tris(hydroxy-methyl) aminomethane (Tris) and hydrochloric acid (HCl), for 14 days, in containers with 45 mL of the incubation medium at 37 °C. The incubation medium was changed every 48 h. After incubation, the samples were rinsed with distilled water to remove any traces of salts from the surface and dried at 40 °C for 24 h. The composition of SBF1x is presented below: Na^+: 142.19 mM, Ca^{2+}: 2.49 mM, Mg^{2+}: 1.5 mM, HCO_3^-: 4.2 mM, Cl^-: 141.54 mM, HPO_4^{2-}, 0.9 mM, SO_4^{2-}: 0.5 mM, K^+: 4.85 mM [20-23]. The presence of mineral crystals onto the surface of the grafted BC was evaluated by SEM analysis. The Ca/P molar ratio was investigated by EDS spectroscopy. SEM analysis has been performed using a QUANTA INSPECT F SEM device equipped with a field emission gun (FEG) with a resolution of 1.2 nm and with an X-ray energy dispersive spectrometer (EDS).

Grafting system	Gravimetric ratio
$(NH_4)_2Ce(NO_3)_6$/BC	5/1
HEMA/BC	4/1
HEMA/BC	7/1
IA/BC	4/1
IA/BC	7/1
AMPSA/BC	4/1
AMPSA/BC	7/1
HEMA-IA/BC	4/1
HEMA-IA/BC	7/1
HEMA-AMPSA/BC	4/1
HEMA-AMPSA/BC	7/1

Table 1
REACTION RECIPES

FT-IR spectra for the BC grafted with synthetic polymers were taken on a Jasco 4200 spectrometer equipped with a Specac Golden Gate attenuated total reflectance (ATR) accessory, using a resolution of 4 cm^{-1} and an accumulation of 60 spectra, in the 4000–600 cm^{-1} wavenumber region.

For biological tests macrophage cell line was employed. The macrophage is considered to be an important cell in the initial non-specific host response against biomaterials [24-25]. Macrophages are responsible of the elimination of foreign bodies in the organism.

A combination approach, MTT and lactate dehydrogenase (LDH) assays, was used to provide valuable information about cell viability and possible cytotoxic effects of the analyzed materials. The specimens used in this study were sterilized by 12h exposure to UV per side and then washed in culture medium for 24h at 4°C. To evaluate the cytotoxicity of potential released compounds, the samples were immersed in culture medium and incubated in standard culture conditions (humidified atmosphere of 5% CO_2 - 95% air at 37°C). After 24h, extracts containing medium (EM) were collected and added undiluted over cells. Phase contrast microscopy was used every 24h, to examine the cell morphology evolution in contact with EM collected from specimens used.

Results and discussions

Swollen bacterial cellulose membranes provided by National Institute for Chemical Pharmaceutical Research and Development are shown in figure 4. Dried samples were obtained at 30 °C for 48 h (fig. 5).

All reactions presented important yields, with values in the range of 50% and 100%, these results indicating copolymerisation reactions. The use of a ACN as specific catalyst for this grafting reaction eliminates the formation of homopolymers poly(itaconic acid), poly(2-hydroxyethyl methacrylate) or poly(2-acrylamido-2-methylpropane sulphonic acid) or their corresponding copolymers.

The grafting mechanism is shown in figure 6 for grafting of itaconic acid onto BC surface and it consists first in the generation of active centres onto the bacterial cellulose surface under inert atmosphere. These radicals are stable in nitrogen medium even 24 h (proved by ESR technique). The reaction proceeds with monomer addition in the

Fig.4. Swollen bacterial cellulose obtained from Acetobacter xylinium DSMZ

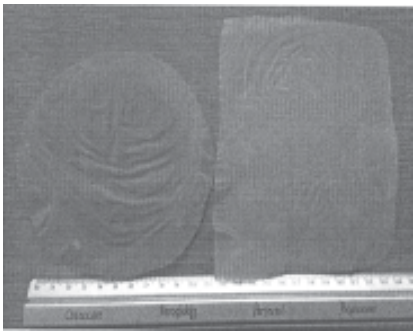

Fig.5. Dried bacterial cellulose obtained from Acetobacter xylinium DSMZ

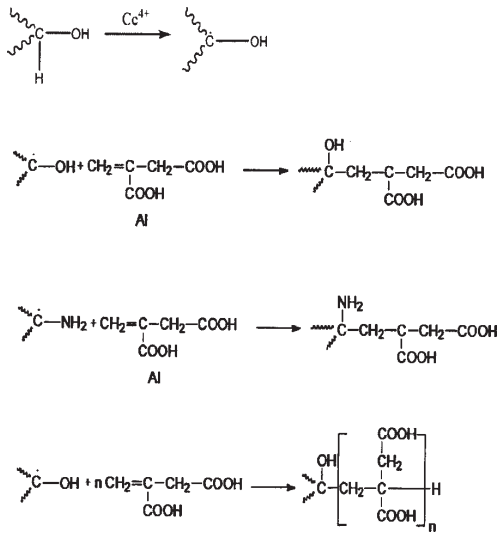

Fig.6. Grafting mechanism of monomers onto the surface of bacterial cellulose

reaction medium followed by propagation and finally termination.

FTIR-ATR analysis was performed on BC membranes by comparison with grafted BC. The BC spectrum revealed the presence of characteristic peaks for cellulose at 3338 cm^{-1} (OH), 2970 and 2895 cm^{-1} (CH_2), 1371 cm^{-1} (CH), 1158 cm^{-1} (C-O-C), 1105 cm^{-1} (C-C), 1052 cm^{-1} (C-O). The spectra of grafted BC with (HEMA-AMPSA) and (HEMA-IA) show a sharp peak at 1709 cm^{-1} characteristic for C=O group and a peak at around 580 cm^{-1} characteristic for sulphonic group.

The main requirement for an artificial material to bond the living bone tissue represents the formation of hydroxyapatite on its surface when implanted in the living bone. To test the ability to induce the formation of hydroxyapatite, one may resort to the use of a solution (SBF, simulated body fluid) that mimics only the inorganic composition of human body fluids.

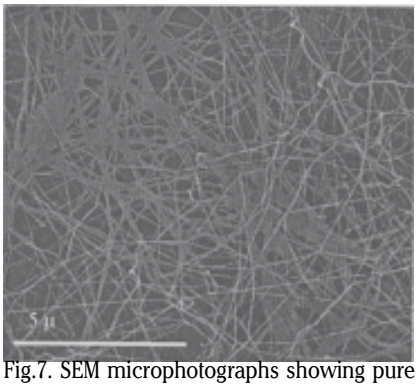

Fig.7. SEM microphotographs showing pure bacterial cellulose membranes immersed in SBF1x

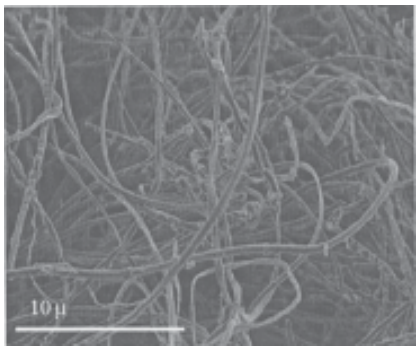

Fig.8. SEM microphotographs showing BC/IA membranes (1/4) immersed in SBF1x

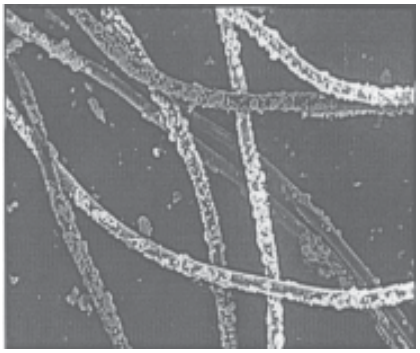

Fig.9. SEM microphotographs showing BC/HEMA-AMPSA membranes (1/7) immersed in SBF1x

The biomineralization capacity of pure BC and BC/HEMA, BC/IA, BC/AMPSA, BC/HEMA-AMPSA and BC/HEMA-IA composites immersed in SBF1x for 14 days was assessed through SEM analysis. Pure BC membranes and those grafted with HEMA show no mineralization potential as revealed by SEM microphotographs (fig.7). Few mineral deposits (fig. 8) with irregular shapes were present onto membranes surface of BC/IA and BC/AMPSA specimens and Ca/P molar ratios of 1.01-1.1 were different from those from bone apatite (1.67). In the case of grafted bacterial cellulose with HEMA-AMPSA and HEMA-IA the mineral phase was composed of microglobules type elementary features. A higher content of synthetic polymers led to a more uniform cover of the surface with mineral phase, a higher number of microglobules and the decrease of the their size. The elementary features became needle-like structures embedded within BC/HEMA-AMPSA and BC/HEMA-IA 1/4 and 1/7 gravimetric ratios (fig. 9). EDS analysis clearly identified Ca and P onto the surfaces of grafted bacterial cellulose materials. The Ca/P molar ratios ranged between 1.5-1.7.

Cells with the concentration of $2.5x10^5$ cells/mL and $1.25x10^5$ cells/mL, grown in a 96 well tissue culture plate, are incubated with the yellow MTT solution for approximate 4 h. MTT (5mg/mL in DMEM without phenol-red) was

Table 2
OBTAINED ABSORBANCE RESULTS FOR THE TEST WITH A CELL CONCENTRATION OF 2.5X10^5 CELLS/mL

Itaconic Acid Concentration (mM)	Absorbance at 570 nm
20	0.139
10	0.410
5	0.665
2.5	0.763
1.25	0.774
1.625	0.822
0.312	0.801
0.156	0.780
0.078	0.910
0.03	1.084
Control	1.562

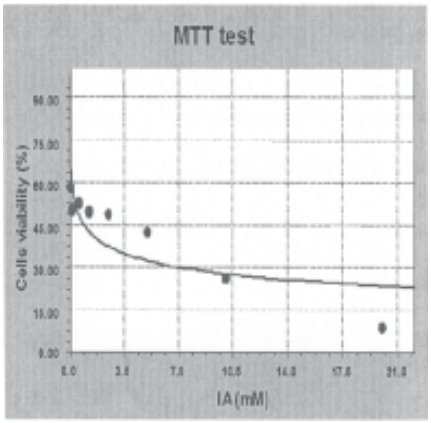

Fig. 10. Cells viability versus itaconic acid concentration for a cell concentration of 2.5x10^5 cells/mL

Table 3
OBTAINED ABSORBANCE RESULTS FOR THE TEST WITH A CELLS CONCENTRATION OF 1.25X10^5 CELLS/mL

Itaconic Acid Concentration (mM)	Absorbance at 570 nm
20	0.101
10	0.531
5	0.825
2.5	0.752
1.25	0.776
1.625	0.905
0.312	1.035
0.156	1.025
0.078	1.114
0.03	1.113
Control	1.582

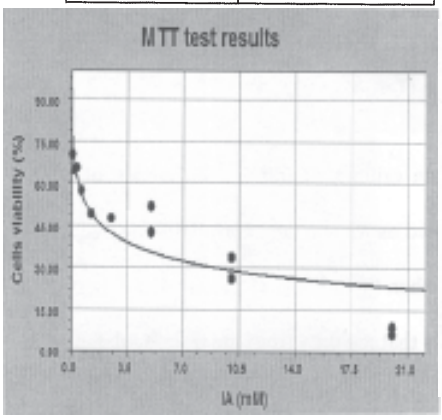

Fig. 11. Cell viability versus itaconic acid concentration for a cell concentration of 1.25x10^5 cells/mL

Table 4
OBTAINED ABSORBANCE RESULTS FOR THE TEST WITH A CELL CONCENTRATION OF 2.5X10^5 CELLS/mL

AMPSA Concentration (µg/ml)	Absorbance at 570 nm
250	0.739
125	0.770
62.5	0.808
31.25	0.781
15.62	0.805
7.81	0.766
3.90	0.684
1.95	0.810
0.97	0.757
0.48	0.803
0.24	0.798
Control	0.824

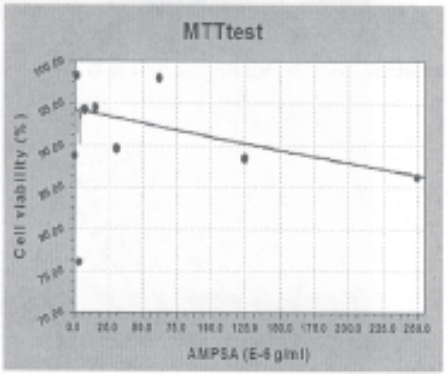

Fig. 12. Cells viability versus AMPSA concentration for a cell concentration of 2.5x10^5 cells/mL

AMPSA Concentration (µg/ml)	Absorbance at 570 nm
250	0.844
125	0.866
62.5	0.961
31.25	0.875
15.62	0.926
7.81	0.923
3.90	0.744
1.95	0.962
0.97	0.878
0.48	0.882
0.24	0.917
Control	0.979

Table 5
OBTAINED ABSORBANCE RESULTS FOR THE TEST WITH A CELLS CONCENTRATION OF 1.25X10^5 CELLS/mL

added to the wells in an amount equivalent to 10% of the culture medium. Itaconic acid was added to the wells in concentrations of: 20 mM, 10 mM, 5 mM, 2.5 mM, 1.25 mM, 0.625 mM, 0.312 mM,.156 mM, 0.078 mM and 0.03 mM. AMPSA was added to the wells in concentrations of 250µg/mL, 125µg/ml, 62.5µg/mL, 31.25µg/mL, 15.62µg/mL,7.81µg/mL, 3.4µg/mL, 1.95µg/mL, 0.97µg/mL, 0.48µg/mL and 0.24µg/mL. Un-treated cells were used as control. These results are shown in tables 2-5 and figures 10-13.

An increase in number of living cells results in an increase in the total metabolic activity in the sample. This increase directly correlates to the amount of purple formazan crystals formed, as monitored by the optical density.

Biological investigations showed that most of the cells retained their typical morphology with more and more extensions (fig. 14). A lot of cells could be evidenced at the

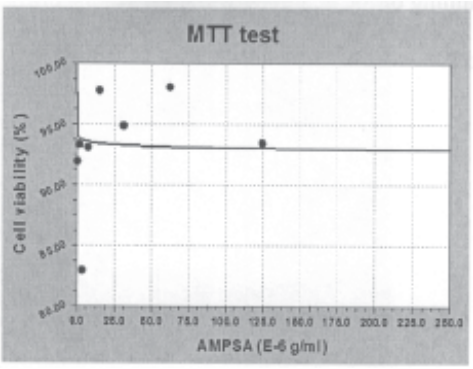

Fig. 13. Cell viability versus AMPSA concentration for a cell concentration of $1.25x10^5$ cells/mL

composite surface. Slightly toxic effects were noticed for grafted BC with 1/7 ratio. Probably the toxic limit concentration of AMPSA and IA for the cells was achieved. On the whole cytototoxicity test have shown that this kind of composites did not show a significant *in vitro* cytotoxicity and could be used for developing biomedical applications.

Conclusions

This research work was focused on the obtaining of composite materials that could initiate the formation of hydroxyapatite crystals. HA is the main mineral part of the bone, so its formation onto the surface or in the polymeric biomaterial after its implantation in the organism, could lead to a better implant tolerance and to the material colonisation with bone cells, which could initiate a bone recovery.

The paper offers an interesting perspective of the obtaining and biomineralization process of composite materials based on biodegradable bacterial cellulose and HEMA-based copolymers with sulphonic and carboxylic groups incubated in SBF1x.

Therefore, the new synthesized materials represent potential scaffolds for apatite crystal growth stimulation in bone tissue engineering.

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References

1.ZAHARIA C., TUDORA, M.R., STANCU, I.C., B. GALATEANU, LUNGU, A., CINCU, C., Materials Science and Engineering C, 32, 2012, p. 945.
2.STANCU, I.C., FILMON, R., CINCU, C., MARCULESCU, B., ZAHARIA,C., TOURMEN, Y., BASLE, M.F., CHAPPARD, D., Biomaterials, 25, 2004, p. 205.
3.VIJAYASEKARAN, S., CHIRILA, T.V., ROBERTSON, T.A., LOU, X., FITTON., J.F., HICKS, C.R., CONSTABLE, I.J., Journal of Biomaterials Science. Polymer Edition, 11, 2000, p. 599.
4.TANG, Z.G., TEOH, S.H., MCFARLANE, W., POOLE-WARREN, L.A., UMEZU, M., Materials Science and Engineering C, 20, 2002, p. 149.
5.YU, NICOLE Y. C., SCHINDELER, A., LITTLE, D.G., RUYS, A.J., Journal of Biomedical Materials Research Part B: Applied Biomaterials, 93B, 2010, p. 285.
6.BIGI, A., PANZAVOLTA, S., ROVERI, N., Biomaterials, 19, 1998, p. 739.
7.BIGI, A., BOANINI, E., PANZAVOLTA, S., ROVERI, N., RUBINI, K., Journal of Biomedical Materials Research, 59, 2002, p. 709.
8.MEHTA, M., SCHMIDT-BLEEK, K., DUDA, G.N., MOONEY, D.J., Advanced Drug Delivery Reviews, 64, 2012, P. 1257.
9.SVENSSON, A., NICKLASSON, E., HARRAH, T., PANILAITIS, B., KAPLAN, D.L., BRITTBERG, M., GATENHOLM, P., Biomaterials, 26, 2005, p. 419.
10.ROHIT KHANNA, KALPANA S. KATTI AND DINESH R. KATTI, Acta Biomaterialia, 7, 2011, p. 1173.
11.GEA, S., TORRES, F.G., TRONCOSO, O.P., REYNOLDS, C.T., VILASECCA, F., IGUCHI, M., PEIJS, T., International Polymer Processing, 22, 2007, p. 497.
12.SOKOLNICKI, A.M., FISHER, R.J., HARRAH, T.P., KAPLAN, D.L., Journal of Membrane Science, 272, 2006, p. 15.
13.CHERIAN, B.M., LEÃO, A.L., S.F. DE SOUZA, THOMAS, S., POTHAN, L.A., KOTTAISAMY, M., Carbohydrate Polymers, 81, 2010, p. 720.
14.CHANG, C., ZHANG, L., Carbohydrate Polymers, 84, 2011, p. 40.
15.PERTILE, R.A.N., ANDRADE, F.K., ALVES JR., C., GAMA, M., Carbohydrate Polymers, 82, 2010, p. 692.
16.CHERIAN, B.M., LEÃO, A.L., S. FERREIRA DE SOUZA, MANZINE COSTA, L.M., G. MOLINA DE OLYVEIRA, KOTTAISAMY, M., NAGARAJAN, E.R., THOMAS, S., Carbohydrate Polymers, 86, 2011, p. 1790.
17.KLEMM, D., SHUMANN, D., UDHARDT, U., MARSCH, S., Progress in Polymer Science, 26, 2001, p. 1561.
18.SOKOLNICKI, A.M., FISHER, R.J., HARRAH, T.P., KAPLAN, D.L, Journal of Membrane Science, 272, 2006, p. 15.
19.OGIWARA, Y., OGIWARA, Y., KUBOTA, H., Journal of Polymer Science, 6, 1968, p. 1489.
20.KOKUBO, T., Acta Metallurgica, 46, 1998, p. 2519.
21.KOKUBO, T., HYUN-MIN KIM, KAWASHITA, M., Biomaterials, 24, 2003, p. 2161.
22.KOKUBO, T., TAKADAMA, H., Biomaterials, 27, 2006, p. 2907.
23.OYANE, A., KAWASHITA, M., NAKANISHI, K., KOKUBO, T., MINODA, M., MIYAMOTO, T., NAKAMURA, M., Biomaterials, 24, 2003, p. 1729.
24.MABILLEAU, G., MOREAU, M.F., FILMON, R., BASLE, M.F., CHAPPARD, D., Biomaterials, 25, 2004, p. 5155.
25.DUMAS, A., GAUDIN-AUDRAIN, C., MABILLEAU, G., MASSIN, P., HUBERT, L., BASLE, M.F., CHAPPARD, D., Biomaterials, 27, 2006, p. 4204.

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