Article

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Hydrogel Bacterial Cellulose Behavior with Stem Cells

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Bacterial cellulose (BC) has become established as a remarkably versatile biomaterial and can be used in a wide variety of applied scientific applications, especially for medical devices. In this work, the bacterial cellulose fermentation process is modified by the addition of hyaluronic acid and chondroitin sulfate (1% w/w) to the culture medium before the bacteria is inoculated. Viability and cytotoxicity study with stem cells using gel bacterial cellulose scaffolds for regenerative medicine are presented at first time. MTT viability assays show higher cell adhesion over time in both gel bacterial cellulose samples, LDH assays showed that both samples has little cytoxicity.

Keywords: Viability and Cytotoxicity Study, Bacterial Cellulose (Nanoskin), Natural Nanocomposites, Regenerative Medicine, Stem Cells.

1. INTRODUCTION

Gluconacetobacter Xylinus (bacterial cellulose, BC) is an emerging biomaterial with great potential in several applications due its high purity, ultra-fine network structure and high mechanical properties in dry state.[1] These features allow its application as scaffolds for tissue regeneration, medical applications and nanocomposites. Some studies have used bacterial cellulose mats to reinforce polymeric matrices and scaffolds with wound healing properties.

BC is a natural cellulose produced by bacterial synthesis by biochemical steps and self-assembling of the secreted cellulose fibrils on the medium. Shaping of BC materials in the culture medium can be controlled by the type of cultivation that changes chain size, origin of strains which produces different proportions of crystalline phase of BC and the kind of bioreactor. BC hydrogel or BC in dry state is then obtained by methods, such as freeze-drying.[2,3] The structural features of microbial cellulose, its properties and compatibility as a biomaterial for regenerative medicine can be changed by modifying its culture medium[4] or surface modification by physical;[5,6] chemical methods[7] and genetic modifications[8] to obtain a biomaterial with less rejection when in contact to the cell and cell interaction.[9,10]

In this scope, cross-linked polysaccharide and glycosaminoglycan hydrogels are used in the synthesis of tissue engineering scaffolds, drug delivery devices, and for cell encapsulation.[11,12] Alginate, agarose, chitosan, chondroitin sulfate (CS), dextran, and hyaluronic acid (HA) are the most commonly used polysaccharides. Biodegradable chondroitin sulfate and hyaluronic acid are used in cartilage tissue engineering, providing cell-interactive matrices for inducing biosynthesis of extracellular matrix (ECM) components for encapsulated chondrocytes and mesenchymal stem cells.[13,14]

Early results also showed that hyaluronic acid (HA) was effective in protecting retinal damage during ophthalmic surgery, reducing scarring, preventing post-operative adhesions and reducing pain while increasing mobility in arthritic joints.[15] In addition, HA also provides important structural support to the extracellular matrix (ECM). Hyaluronan-binding proteins, called hyaladherins, mediate its interaction with various extracellular components, including proteoglycans, collagen and fibrin, which stabilizes both HA and ECM.[16]

However, the success of the scaffold to be used in tissue engineering depends, in part, on the adhesion and growth of cells of interest on its surface. The surface chemistry of the material may define the cellular material and thus affect the adhesion, proliferation, migration and cell function.[17–19]

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Stem cells are a non-specialized cell type, which can self-renew and remain for a long period of time with the potential to derive in a cell lineage or tissue with specialized functions. Tissue-specific stem cells, or adult stem cells, have been considered as an alternative for the use of embryonic stem cells , due to their availability, ease of acquisition and growth.[20–22] The isolation and characterization of mesenchymal stem cells from such an accessible source as the teeth has opened up a new field of research that can be used in the treatment of many clinical conditions in dentistry and medicine. Thus, the study of populations of adult stem cells with plasticity similar to embryonic stem cells has been the target of numerous researchers.

Obtaining stem cells from human exfoliated deciduous teeth (SHED) is both simple and convenient. Miura et al.[23] identified SHED cells as proliferative, capable of differentiation into a variety of cell types like neural cells, adipocytes and odontoblasts. When compared to dental pulp stem cells (DPSC), SHED cells exhibited higher proliferation rates, osteoinductive capacity *in vivo*, results from *in vivo* transplantation suggested that SHED have a greater capability for mineralization than DPSC.[24]

In 2008, Cordeiro et al.[25] suggested the SHED cells may be a valuable cell source for dental pulp tissue engineering. They seeded SHED cells in biodegradable scaffolds prepared within human tooth slices and transplanted into mice. The resulting tissue presented an architecture and cellularity that resembled a dental pulp. Several nanocomposites were tested specifically with stem cell adhesion and differentiation.[26] Cell adherence and proliferation ability of hBMSCs (human bone mesenchymal stem cells) on scaffolds were improved by coating with HA/PLLA nanocomposites.[27] Other nanocomposites and electrospun nanocomposites succeeded in testing the interaction with cells.[28–32]

Olyveira et al.[33] reported at first time adhesion and viability study with human dental pulp stem cell using natural nanotolith/bacterial cellulose scaffolds for regenerative medicine. Pure Bacterial cellulose and bacterial cellulose nanocomposites showed great response with cell essays over time and these results show BC/BC nanocomposite as potential biomaterial for cell delivery applications, mainly because their natural properties and constitution are like the extracellular matrix. Results shows that fermentation process and nanotoliths agglomeration decrease initial human dental pulp stem cell adhesion however tested bionanocomposite behavior has cell viability increase over time.

Olyveira et al.[34] reported at first time adhesion and viability studies with human dental pulp stem cells using natural bacterial cellulose/hyaluronic acid and bacterial cellulose/gelatin as scaffolds for regenerative medicine. MTT viability assays show higher cell adhesion in bacterial cellulose/gelatin and bacterial cellulose/hyaluronic acid scaffolds over time with differences due to fiber agglomeration in bacterial cellulose/gelatin. Confocal microscopy images showed that the cell were adhered and well distributed within the fibers in both types of scaffolds.

In this work, novel studies of natural nanocomposites with bacterial cellulose for functional materials is reported. In order to produce scaffolds with drug delivery ability, porous structure and better cell adhesion, fermentation changes in gel bacterial cellulose with chondroitin sulfate and hyaluronic acid were performed and it's cell behavior is presented.

2. MATERIALS AND METHODS

2.1. Materials

The bacterial cellulose raw material (Nanoskin) was provided from Innovatec's (São Carlos SP, Brazil). Chondroitin sulfate and hyaluronic acid sodium salt from *Streptococcus equi* (bacterial glycosaminoglycan polysaccharide) were purchased from Sigma Aldrich.

2.2. Methods

2.2.1. Synthesis of Bacterial Cellulose and Bacterial Cellulose/Chondroitin Sulfate/Hyaluronic Acid

The acetic fermentation process was achieved by using glucose as a carbohydrate source. Results of this process are vinegar and a nanobiocellulose biomass. The modifying process is based on the addition of hyaluronic acid and chondroitin sulfate (1% w/w) to the culture medium before the bacteria is inoculated. Gel Bacterial cellulose (BC) is produced by Gram-negative bacteria Gluconacetobacter xylinus, which can be obtained from the culture medium in the pure 3-D structure, consisting of an ultra fine network of cellulose nanofibers.[35]

2.2.2. Bionanocomposite Preparation

In the present study, a novel biomaterial has been explored and different bacterial cellulose nanocomposites have been prepared; 1) BC (BC1), 2) BC/chondroitin sulfate/hyaluronic acid (BC2). Both samples were washed and it's medium was changed with cells culture medium.

2.3. Samples of Dental Pulp Tissue from Teeth

In order to isolate the cells from the pulp tissue and establish their culture, dental pulp was removed from teeth in the resorption process. After extraction, the teeth was immersed in 1 mL culture medium DMEM/Hepes (Sigma Aldrich), 10% fetal bovine serum (GIBCO), 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco) and 0.45 μg/mL gentamicin (Gibco) at room temperature for transport to the laminar flow.

2.3.1. Cell Culture

Cell suspension in the culture medium was seeded onto a 12 well culture plate and then incubated at 37 °C in a

humidified atmosphere of 5% CO_2.[36] The culture medium was changed 24 hours after initial plating and then every 3 days thereafter. The culture was maintained under these conditions until confluence of approximately 90% when it was then held in its first passage. The cells in culture were harvested with trypsin-EDTA solution 0.5% (Sigma-Aldrich) and transferred to sub-cultures in their culture medium. The sub-culture was maintained in a monolayer until required for the next raise. When the cells reached approximately 90% of confluence between the 5th passage (P5), cell viability was assessed with trypan blue 4% (Gibco) in a Neubauer chamber and testing, to verify the interaction between cells and hydrogel, was performed as follows.

2.4. Characterization

Transmission infra-red spectroscopy (FTIR, Perkin Elmer Spectrum 1000). Influences of hyaluronic acid and chondroitin sulfate in bacterial cellulose were analyzed in the range between 250 and 4000 cm^{-1} and with 2 cm^{-1} resolution with samples.

2.4.1. Cell Viability

For the study of cell viability during the 7 day of culture, as performed for the cell adhesion essay, the cells were seeded onto each type of hydrogel in triplicate and then incubated at 37 °C in a humidified atmosphere of 5% CO_2. To collect the initial viability of the seeded cells, the viability of 5×10^4 cells was analyzed, 6 hours after seeding onto the culture dishes. Analysis was then made after the start of the cultivation of the cells in the biomaterial. After each trial, cell viability was performed by the salt tretazolyum method, a colorimetric assay using bromide 3-(4,5-dimethylthiazol-2-yl)-2,5–diphenyltetrazolium bromide (MTT). After the experiment time, the culture medium was removed and 200 μL MTT solution (0.25 mg/mL) was added and maintained for 2 hours. The MTT was then removed and 200 μL of dimethyl sulfoxide (DMSO) was added to dissolve the crystals formed by the reaction. Using 96-well plates, the absorbance of the final solution was analyzed by a spectrophotometer (Wallac EnVision-Perkin Elmer). The data was calculated using the difference in absorbance between the wavelengths (560 nm–630 nm). As a control group, the cells were seeded in a similar way onto 24-well plates (in triplicate) without hydrogel and maintained by the same experimental period and the same procedures for data collection were performed.

Cell Cytotoxicity-In the supernatant of cell cultures, cell cytotoxicity is assessed by the determination of the enzyme lactate dehydrogenase (LDH) in the machine 560 LabMax. In thermostated cuvettes (cups) are added approximately 0.2 ml of sample. In this test, the leakage of cytoplasmic LDH enzyme and its presence is measured in the culture medium in which cells are cultured and it is indicative of damage to the cell membrane. Increasing the dosage of this enzyme increases in cell death. The materials were assayed in 7 days, in triplicate for a minimum of four separate experiments with comparable results.

3. RESULTS AND DISCUSSION

3.1. FTIR-Interaction Between Bacterial Cellulose with Hyaluronic Acid and Chondroitin Sulfate

Influences of hyaluronic acid (HA) and chondroitin sulfate (CS) in bacterial cellulose were analyzed in the range between 250 and 4,000 cm^{-1} and with resolution of 2 cm^{-1} with FTIR analysis. The main features of the bacterial cellulose in infrared spectroscopy is: 3,500 cm^{-1}: OH stretching, 2,900 cm^{-1}: CH stretching of alkane and asymmetric CH_2 stretching, 2,700 cm^{-1}: CH_2 symmetric stretching, 1,640 cm^{-1}: OH deformation, 1,400 cm^{-1}: CH_2 deformation, 1,370 cm^{-1}: CH_3 deformation, 1,340 cm^{-1}: OH deformation and 1,320–1,030 cm^{-1}: CO deformation.[37]

It can be observed similar OH stretching (at 2,900 cm^{-1}) in bacterial cellulose/hyaluronic nanocomposites (BC/HA) and chondroitin sulfate nanocomposites (BC/CS), mainly because of the NH_2 interaction with hydroxyl groups (Fig. 1). Besides, changes can be observed in the symmetrical stretching of CH_2 bonds of bacterial cellulose structures at the absorption peak of 1,640 cm^{-1}. Another absorption peak was obtained in the range of 1,490 cm^{-1} on both samples, which shows the presence of a carbonyl group in the bacterial cellulose together with bonds corresponding to those of glycoside, including C–O–C at 1,162 cm^{-1} (as in the case of natural cellulose).[35] These results clearly show one possible interaction between bacterial cellulose and chondroitin sulfate/hyaluronic acid, mainly by hydrogen interactions between hydroxyl and carbonyl groups.

3.2. Cell Viability

For the successful application of scaffolds/hydrogel in tissue engineering, a crucial feature is that the matrices

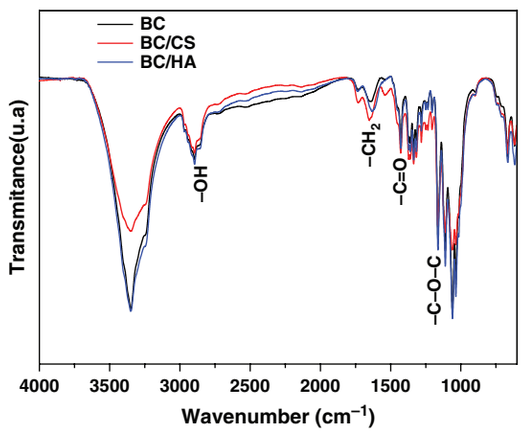

Figure 1. FTIR spectra of bacterial cellulose nanocomposites.

promote cell adhesion. According to Andrews and colleagues,[38] cell adhesion is mediated by the adsorption of extracellular matrix proteins produced by cells on the surface of the scaffold. The signalling pathways are then activated and cell adhesion occurs in the mould by means of receptors. Therefore, accommodation and cell behavior is strongly affected by the structure of the scaffolds/hydrogel and cell adhesion assay becoming important in order to determine whether the scaffolds/hydrogel have a good structure for the initial interaction with cells.

The metabolic activity was assessed by measuring the activity of the enzyme mitochondrial succinate dehydrogenase (MTT assay), which is widely used in *in vitro* evaluation of cell viability.[39, 40]

Through the MTT assay, it was found that after 7 day of experiment, the three groups (samples I and II and control) showed similar behavior, with no statistical difference. The control group had an absorbance greater than the two test groups, indicating greater proliferation of cells in the control group. The test groups maintained with similar absorbance until end of day, indicating that the number of cells remained constant-which is nonetheless a good result (Fig. 2). This shows that the fibers prepared for the study provide an initial adhesion and increase of viability in the initial stage and that they have ability to promote maintenance of viability in the long-term.

3.3. Cell Cytotoxicity

Cell adhesion and proliferation of different types of cells onto various surfaces depends on polymer surface characteristics like wettability,[41, 42] surface charge[43] and surface free energy and topography.[44]

According to the graph below, the sample with cytotoxicity was control sample. Cells grown on BCs released the same doses of LDH than control (cells cultured directly on the well) without indication of cytotoxicity. The viability detected was similar to control and cytotoxicity as well.

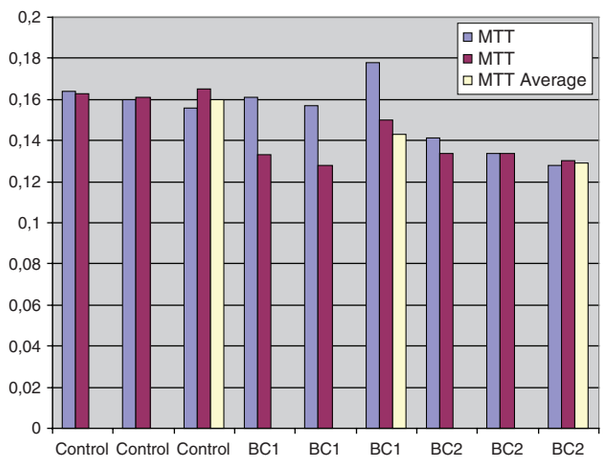

Figure 2. Cell viability assay over a time period of 7 day in gel bacterial cellulose (sample BCI); gel bacterial cellulose/hyaluronic acid/chondroitin sulfate (sample BCII), control group.

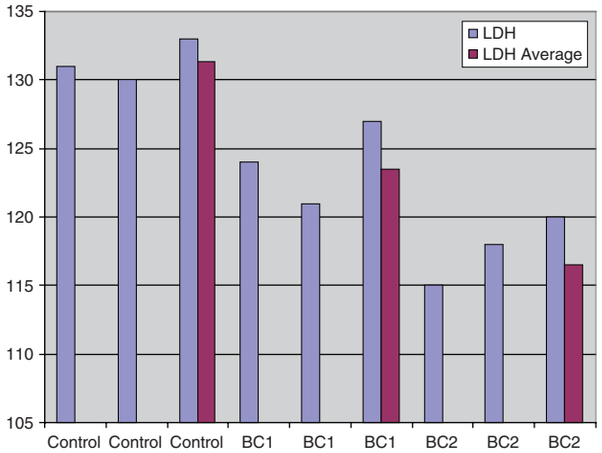

Figure 3. Cell cytotoxicity over a time period of 7 day in gel bacterial cellulose (sample BCI); gel bacterial cellulose/hyaluronic acid/chondroitin sulfate (sample BCII), control group.

The difference in cell viability and cytotoxicity, may be attributed to the differences in their surface properties. However, other factors can affect cellular viability. Hydrophilicity, on its own is neither necessary or suffcient for cell viability. Chemical properties such as carboxyl,[45] hydroxyl[46, 47] groups and topography can be important in cell attachment and growth depending on the type of cell, however it can be observed little difference between samples BC1 and BC2.

4. CONCLUSION

Bacterial cellulose was successfully modified by changing the fermentation medium as shown by FTIR, which produced scaffolds with different surface morphology but similar cell viability, attachment and cytotoxicity. Natural scaffolds with bacterial cellulose and bacterial cellulose nanocomposites had good cell adhesion over time between tested samples, being an extremely effective material for tissue regeneration. However, a better controlled development in methods for production, fermentation and topography control is essential for better surface morphology with higher adhesion and viability cells to widespread use of these hydrogels. Thus, undoubtedly, natural-origin polymers or nature-inspired materials appear as the natural and desired choice for medical applications.

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