Effect on viability and cellular proliferation of rhBMP-2 immobilized on TEMPO modified cellulose hydrogel

Efeito na viabilidade e proliferação celular de rhBMP-2 imobilizada em hidrogel de celulose modificado por TEMPO

Efecto sobre la viabilidad celular y proliferación de rhBMP-2 inmovilizada en hidrogel de celulosa modificado con TEMPO

Ana Lucia Colange
ORCID: https://orcid.org/0000-0001-5017-0364
Universidade de Araraquara, Brazil
E-mail: anacolange@hotmail.com
Carlos Sabino de Oliveira
ORCID: https://orcid.org/0000-0001-9920-0106
Universidade de Araraquara, Brazil
E-mail: carlos.olvr@outlook.com
Benedito Domingos Neto
ORCID: https://orcid.org/0000-0001-7607-2738
Universidade de Araraquara, Brazil
E-mail: bdneto@uniara.edu.br
Heloisa Sobreiro Selistre de Araújo
ORCID: https://orcid.org/0000-0002-2372-7814
Universidade Federal de São Carlos, Brazil
E-mail: hsaraujo@ufscar.br
Eliane Trovatti
ORCID: https://orcid.org/0000-0002-0495-8115
Universidade de Araraquara, Brazil
E-mail: elianetrov@yahoo.com.br
Monica Rosas da Costa Iemma
ORCID: https://orcid.org/0000-0003-1173-2111
Universidade de Araraquara, Brazil
E-mail: mrciemma@uniara.edu.br

Abstract
BMP´s are signaling proteins that belong to the Transforming Growth Factor-β (TGF-β) superfamily. These proteins promote the recruitment and differentiation of mesenchymal progenitor cells into bone forming cells, the osteoblasts and increase the rate of bone formation. The carrier systems to release rhBMP-2 to the action site are based on the use of free and soluble BMP incorporated into biopolymers such as collagen, gelatin, chitosan, hyaluronic acid and silk. The fused rhBMP-2-thioredoxin could be an interesting approach for new advances in the field of carrying systems of these growth factors. The fused protein thioredoxin can be useful as a coupling agent of BMP-2 to the carrier system, binding it to the surface of the matrix and it is one of the main aims of this work. The recombinant protein rhBMP-2 was produced by IPTG induction obtaining a soluble protein without the need for refolding process. The immobilization of rhBMP-2 at the surface of the TEMPO modified cellulose nanofibrils was indicated by FTIR spectroscopy. The cellular viability tests indicated increased proliferative behavior of both, C2C12 and stem cells from rats, when seeded in presence of rhBMP2 when compared to the free rhBMP2 substrate. The calcified extracellular matrix confirmed the increased activity of the rhBMP2-cellulose substrate, indicating the success of the proposed method. The cell proliferation assays indicated the method used to immobilize rhBMP2 onto the surface of the TEMPO modified cellulose was successful. The cells growth increased when compared to the reference sample free of rhBMP2.
Keywords: rhBMP2; TEMPO modified cellulose nanofibrils; Immobilization; Grafting; Cell proliferation.

Resumo
As BMP's são proteínas sinalizadoras que pertencem à superfamília Fator de Crescimento Transformador Beta (TGF-β). Essas proteínas promovem o recrutamento e a diferenciação de células progenitoras mesenquimais em células formadoras de osso, os osteoblastos e aumentam a taxa de formação óssea. Os sistemas carreadores para liberação de rhBMP-2 no local de ação são baseados no uso de BMP livre e solúvel incorporada em biopolímeros como colágeno,

gelatina, quitosana, ácido hialurônico e seda. A rhBMP-2-tioredoxina fundida pode ser uma abordagem interessante para novos avanços no campo de sistemas de transporte desses fatores de crescimento. A proteína fundida tiorredoxina pode ser útil como agente de acoplamento da BMP-2 ao sistema carreador, ligando-a à superfície da matriz e é um dos principais objetivos deste trabalho. A proteína recombinante rhBMP-2 foi produzida por indução de IPTG obtendo-se uma proteína solúvel sem a necessidade de processo de redobragem. A imobilização de rhBMP-2 na superfície das nanofibrilas de celulose modificadas por TEMPO foi indicada por espectroscopia FTIR. Os testes de viabilidade celular indicaram aumento do comportamento proliferativo tanto de C2C12 quanto de células-tronco de ratos, quando semeadas na presença de rhBMP2 quando comparadas ao substrato rhBMP2 livre. A matriz extracelular calcificada confirmou o aumento da atividade do substrato rhBMP2-celulose, indicando o sucesso do método proposto. Os ensaios de proliferação celular indicaram que o método usado para imobilizar rhBMP2 na superfície da celulose modificada por TEMPO foi bem-sucedido. O crescimento das células aumentou quando comparado com a amostra de referência isenta de rhBMP2.

Palavras-chave: rhBMP2; Nanofibrilas de celulose modificadas por TEMPO; Imobilização; Enxerto; Proliferação celular.

Resumen

Las BMP son proteínas de señalización que pertenecen a la superfamilia del factor de crecimiento transformante beta (TGF-β). Estas proteínas promueven el reclutamiento y la diferenciación de células progenitoras mesenquimales en células formadoras de hueso, osteoblastos, y aumentan la tasa de formación de hueso. Los sistemas portadores para la liberación de rhBMP-2 en el sitio de acción se basan en el uso de BMP libre y soluble incorporado en biopolímeros como colágeno, gelatina, quitosano, ácido hialurónico y seda. La rhBMP-2-tioredoxina fusionada podría ser un enfoque interesante para futuros avances en el campo de los sistemas de transporte de estos factores de crecimiento. La proteína fusionada con tiorredoxina puede ser útil como agente de acoplamiento de BMP-2 al sistema transportador, uniéndola a la superficie de la matriz y es uno de los principales objetivos de este trabajo. La proteína recombinante rhBMP-2 se produjo por inducción de IPTG obteniendo una proteína soluble sin necesidad de un proceso de replegamiento. La inmovilización de rhBMP-2 en la superficie de las nanofibrillas de celulosa modificadas con TEMPO se indicó mediante espectroscopia FTIR. Las pruebas de viabilidad celular indicaron un aumento en el comportamiento proliferativo de las células madre C2C12 y de rata, cuando se sembraron en presencia de rhBMP2 en comparación con el sustrato rhBMP2 libre. La matriz extracelular calcificada confirmó el aumento de la actividad del sustrato de celulosa rhBMP2, lo que indica el éxito del método propuesto. Los ensayos de proliferación celular indicaron que el método utilizado para inmovilizar rhBMP2 en la superficie de celulosa modificada con TEMPO fue exitoso. El crecimiento celular aumentó en comparación con la muestra de referencia sin rhBMP2.

Palabras clave: rhBMP2; Nanofibrillas de celulosa modificadas con TEMPO; Inmovilización; Injerto; Proliferación celular.

1. Introduction

BMP´s are signaling proteins that belong to the Transforming Growth Factor-β (TGF-β) superfamily (Wang et al., 2014). These proteins promote the recruitment and differentiation of mesenchymal progenitor cells into bone forming cells, the osteoblasts (Okamoto et al., 2006) and increase the rate of bone formation. The increase of rate of bone formation can overcome the limited availability of bone, namely the autologous bone, in critical bone defects or at the site of the surgeries. The approaches to supply the demand for bone formation and repairing represent disruptive technologies in orthopedics and dentistry, once the success of these fields lies on the bone healing approaches (Yu et al., 2010). In this vein, BMP´s, mainly BMP-2 and BMP-7, reached a noble status because of its bone healing capability, and have been used commercially for clinical applications (Gautschi et al., 2007) rhBMP-2, marketed as InFUSEVR and rhBMP-7, marketed as OP-1VR are commercial products based on growth factor. Although, few approaches are available for administration of such growth factors (Khojasteh et al., 2013; Nandi et al., 2010; Xiao et al., 2007).

The action of BMPs depends on its binding to specific membrane receptors, the type-I and type-II membrane serine/threonine receptors, to stimulate the initiation of the intracellular signaling cascade of effects (Dimitriou & Giannoudis, 2005; Termaat et al., 2005). The availability of BMP-2 at the action site is crucial for its therapeutic effect, therefore its production and availability still remain a drawback. The expression of the human genes in microorganism systems has been one of the alternatives to overcome the low availability of this (and other) protein. Many human proteins for use in the biomedical field are synthesized by bacteria, however, they may fail to accumulate at high levels and can be displayed

insoluble, in the form of inclusion bodies within the cells (LaVallie et al., 1993). High levels of active and soluble BMP´s is normally produced linked to the fusion protein thioredoxin (LaVallie et al., 1993). Breaking the linkage between the thioredoxin and BMP´s and the purifying process increases the time and the production costs, however it is normally performed because the free pure protein finds commercial application and can be used in several approaches to stimulate the cells proliferation and tissues repairing.

The carrier systems to release rhBMP-2 to the action site are based on the use of free and soluble BMP incorporated into biopolymers such as collagen, gelatin, chitosan, hyaluronic acid and silk (Agrawal & Sinha, 2017; Ruhé et al., 2006). The use of BMP-2 covalently attached to the surface of a polymeric matrix (silk fibroin) was already studied, leading to the differentiation of bone marrow stromal cells into bone (Karageorgiou et al., 2004). The advantage of the use of immobilized protein to the matrix is its longer residence time at the site of action. Nevertheless, the fused rhBMP-2-thioredoxin could be an interesting approach for new advances in the field of carrying systems of these growth factors. The fused protein thioredoxin can be useful as a coupling agent of BMP-2 to the carrier system, binding it to the surface of the matrix and it is one of the main aims of this work.

Thioredoxin is a thiol rich protein. Thiol or sulfhydryl groups (-SH) are chemical groups with high affinity to covalently bind to double bonds, specially with maleimide bonds. The linkage of SH groups to maleimide is a classical click reaction activated by UV light, fast and efficient (Algar, 2017). TEMPO oxidized cellulose hydrogel (ToCNF) was the substrate of choice for this coupling because the oxidation generates carboxylic groups at the fibers surface capable of reacting with the primary amino functions. These amino groups can be used to attach a maleimide functionality to the surface of the nanofibers, which represents the binding site for the thiol groups from thioredoxin. ToCNF also represents a born material with potential for cell encapsulation and release for application in regenerative medicine, besides its biocompatibility and disintegration in physiological environment. Thus, its coupling with the rhBMP-2 protein can open new perspectives of use as a support for protein carrying, besides to test the capability of the BMP-thioredoxin to stimulate cell proliferation.

2. Materials and Methods

2.1 Materials

Sodium hypochlorite (NaClO, 2-2.5 % available chlorine) was purchased from Anidrol and folic acid (pharmaceutical grade) was kindly donated by a local pharmacy. The sugar cane bagasse was supplied by a producing farm. Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (Nutricell) purchased from (Gibco/Thermofisher), penicillin and streptomycin 10 U/mL (Sigma Aldrich), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium salt (MTT) from Merck, isopropyl alcohol, dimethyl sulfoxide, sodium hydroxide, sodium hypochlorite (NaClO) and phosphate buffer saline (PBS) purchased from Synth, trypsin from Gibco and the deuterated solvents were purchased from Sigma-Aldrich. Dithiotreitol, Sigma All other chemicals were purchased from Sigma-Aldrich Brazil Ltd. (São Paulo) and were used as received. Ni-NTA Sepharose resin, isopropyl-β-D-thiogalactoside (IPTG), of β- 2-Mercaptoethanol, PBS1X (Phosphate Buffered Saline, BCA (Ácido Bicinconínico), BCIP (5-bromo-4-chloro-3-indolyl-phosphate) is used in conjunction with NBT (nitro blue tetrazolium).

2.2 Methods

2.2.1 rhBMP-2 production

The plasmid pET32rhBMP-2 (rhBMP-2 gene carrier) was introduced in E. coli Rosett (DE3) pLysS aiming the expression of the recombinant protein rhBMP-2. The transformed bacteria was cultivated overnight in 10 mL of LB medium with 100 μg/mL of ampicillin. The grown culture (10 mL) was used to inoculate 250 mL of LB medium with 100 μg/mL of

ampicillin medium and incubated until reaching the O.D.660nm ≈ 0.6 - 0.7. A 1 mL aliquot was withdrawn from the culture medium (T0 = zero time point before induction) and centrifuged at 13.000 rpm for 5 min at room temperature. The supernatant was removed and the pellet was resuspended in sample buffer (125 mM de Tris-HCl, pH 6,8; 4% SDS; 20% glycerol; 1% β-mercaptoethanol; 0,03 mM bromophenol)

The samples were stored for electrophoresis in polyacrylamide gel analysis (SDS-PAGE). The induction of the rhBMP-2 expression was performed adding 1mM IPTG in the culture medium at 250 rpm, 20 °C for 16 h. After the induction period, 1 mL was withdrawn and processed for SDS-PAGE as previously described. The remaining culture medium was centrifuged at 5.000 rpm for 20 min at 4 °C (Sorvall® RC 5C Plus centrifuge), the supernatant was removed and the cells were resuspended into 12,5 mL of buffer A (10 mM Imidazole diluted in PBS Buffer). The cell suspension was sonicated in a ultrasonicator (marca, modelo; 6 cycles 1 min amplitude at 20 % followed by 30 sec resting) for lysis. The cells lysate was centrifuged at 15.000 rpm for 20 min at 4 °C (Sorvall® RC 5C Plus). 50 μL from the soluble and 50 μL from insoluble fraction were treated with β-mercaptoethanol buffer for SDS-PAGE analysis.

2.2.2 rhBMP-2 purification

The soluble fraction of the cell lysate prepared in item 2.2.1, was purified using a 1 mL nickel Immobilized-metal Affinity Chromatography (IMAC,Quiagen) column in gravity flow previously equilibrated with 10 CV of buffer A. For such, 5 mL of the soluble fraction was applied into the resin and after sedimentation the unbounded protein was removed. Then, the resin was washed using buffer A 20 CV, followed by the protein elution using buffer B (buffer A plus imidazole 250 mM and 500 mM), 2 CV. Each sample was analyzed by SDS-PAGE. The rhBMP-2 purified fractions were inserted into a dialysis bag in PBS1X (Phosphate Buffered Saline) to eliminate the imidazole. The protein content was determined by the BCA method.

2.2.3 Immunodetection

After electrophoresis (item 2.2.2.), the separated proteins were transferred onto a nitrocellulose membrane using the semi-dry transfer system Trans Blot SD Semi-Dry Transfer Cell Bio- Rad. Then, the membrane was blocked (non specific sites) using TBS 1X (Tris-HCl 100 mM pH 7,5, NaCl 150 mM) plus 5% (vol) skimmed milk overnight followed by washing (three times) with TBS 1x plus Tween-20 0,05 % (vol), for 5 min. The membrane was incubated with the monoclonal conjugated antibody Anti-polyHistidine – Alkaline-Phosphatase conjugated, anti-mouse (Sigma-Aldrich, St. Louis, MO, EUA), 1:2000 in TBS for 2 h. The membrane was then washed three times with TBS plus Tween, under gentle stirring for 5 min and relieved in AP-buffer (alkaline phosphatase buffer: Tris-HCl 0,1 M pH 9,5, NaCl 0,1M, MgCl2 5 mM) plus BCIP conjugated with NBT for the colorimetric detection of alkaline phosphatase activity. Deionized water was added to stop the reaction.

2.2.4 Cell proliferation assays

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is based on the conversion of MTT to formazan. This conversion occurs only in the presence of viable cells, whose mitochondrial activity can reduce the tetrazolium salt (yellow), forming lipophilic formazan crystals, which upon solubilization produce a strong violet-colored solution. This violet staining allows the measurement of absorbance by spectrometry, providing information on the amount (percentage) of viable cells (Kumar et al., 2018).

The C2C12 cell line is a subclone of the rat myoblast cell line and was used to prove the biological activity of rhBMP-2 produced in this work. This choice was based on a report in the literature that shows that the C212 lineage has receptors for rhBMP-2 since a change in the differentiation pathway from myoblast to osteoblast was detected when cultured in the presence

of rhBMP-2 (Katagiri et al., 1994).

C2C12 cells were incubated in DMEM culture medium supplemented with fetal bovine serum (FBS 10 % vol), penicillin 100 U/mL and streptomycin 0.1 mg/mL. The cells (5x103 cells/well,) were seeds in a 96 well plate, at 37°C in 5 %CO2 atmosphere. After 24 hours to allow the adhesion, the medium was removed and cells were treated with different concentrations of rhBMP-2 (0.15 µg, 0.3 µg, 0.5 µg and 150 µg/well) in DMEM 0,5% FBS. and incubated at 37°C in a 5 % CO2 atmosphere for 24 hours and control were cells treated only with DMEM 0,5% FBS. After the incubation period, the culture medium was removed and cell viability tests (MTT assay) were carried out. A volume of 50 µL of MTT reagent (0.05 mg/mL in PBS 1X) was added to each well and the microplates were incubated at 37°C for 4 h. The obtained formazan crystals were solubilized in isopropyl alcohol (100 uL/well) and the absorbance for each well was read at 570 nm in a microplate reader Spectramax i3 (Molecular Devices) spectrophotometer. Two different experiments were performed in triplicate. The cell viability was reported in percentage of viability.

2.2.5 Preparation of TEMPO-modified nanofibrillated cellulose (ToCNF)

Cellulose was obtained by process described in detail according to a published study (Trovatti et al., 2017).

Ten grams of sugar cane bagasse fibers (lower than 1 mm) were washed in a Soxlet system with toluene/ethanol 2:1 (v/v) and dried at room temperature. The fibers were dispersed in 1.3% sodium chlorite solution (200 mL), pH ~4 and stirred for 1 h at 75 ºC using a reflux condenser, followed by washing with deionized water up to neutral pH. The fibers were then treated with 2 % (wt) KOH aqueous solution and stirred at 85 ºC for 2 hours. The fibers were washed with deionized water up to neutral pH and the both last procedures were repeated. The bleached sugar cane bagasse pulp was oxidized with 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) following a well-established method previously described by Saito et al. (2007). Briefly, to a dispersion of pulp in water (3.3 %w) sodium bromide (1.2 mmol/g cellulose) and TEMPO reagent (0.08 mmol/g cellulose) were added, followed by the slow addition of sodium hypochlorite (13 mL/g cellulose); the suspension was maintained at pH 10 (with 0.5 M NaOH, until stable) under stirring (4 h). The modified cellulose was washed (0.60 mm membrane) with deionized water to neutral pH. The oxidized fibers were homogenized using a kitchen blender and then, sonicated in an ultrasonicator until the formation of a transparent gel (about 20 min, ice bath). The content of carboxylic groups was determined by conductometric titration.

2.2.6 Synthesis of the furan-protected amino maleimide (FPAM) and its coupling to ToCNF

The synthesis of the FPAM and its coupling to ToCNF were carried out following the method previously described (Trovatti et al., 2017). Briefly, furan-maleic anhydride adduct or Diels Alder adduct 7 (3.32 g, 0.02 mol) was dissolved in anhydrous methanol (30 mL) and mixed with hexamethylenediamine (2.32 g; 0.02 mol). The system was stirred under reflux for 8 h and left overnight to cool to room temperature. The precipitate (FPAM) was washed with methanol and dried. For coupling, the suspension of ToCNF (40 g; 0.15 %wt), EDC (58 mg; 0.37 mmol), NHS (33 mg; 0.28 mmol) and FPAM (66 mg; 0.25 mmol) were mixed and stirred for 2 h at room temperature in capped erlenmeyer. The suspension was filtered (0.6 µm pore membrane) and washed with distilled water. Finally, the removal of furan was carried out by heating the modified ToCNF suspension for 2 h in an oil bath (110 ºC), yielding N-hexamethylamine maleimide functionalized ToCNF (AM-ToCNF).

2.2.7 Conjugation of the thiolated protein to the maleimide from the AM-ToCNF

The protein was treated with dithiothreitol (DTT) for reducing the native disulfide bonds in and preventing their oxidation to the disulfide state before reaction with AM-ToCNF. For such, 100 µL of the protein solution (1.4 µg/mL) was mixed with 1.2 µL of DTT solution (1mM) and stirred for 30 min at 40 ºC. The suspension was dialyzed for 2 h using a tube

dialysis (14 KDa).

The protein suspension (100 µL, 1.4 µg/mL) was mixed with 1 g of aqueous AM-ToCNF (0.5 % wt) suspension in a quartz flask and stirred under 365 nm UV light for 20 min, followed by further stirring at room temperature for 24 h. The suspension was filtered and the filtrate was separated for analysis, then, the sample was extensively washed with deionized water in order to remove free protein.

2.2.8 Characterization of the reaction products

Fourier-transform infrared (FTIR) spectra were acquired with a PerkinElmer Spectrum 100 using an ATR accessory, with 4 cm-1 of resolution, 16 scans, from 650 to 4000 cm-1.

2.2.9 Cell proliferation at the surface of ToCNF-rhBMP2

Cell proliferation at the surface of ToCNF-rhBMP2 – the ToCNF-rhBMP2 hydrogel (50 mg, 1 %wt) was used to coat the surface of the wells (96 well plates). MC3T3 cells (5×10^3 cells/well) were added to the surface of the hydrogel and incubated in DMEM supplemented culture medium at 37 ± 2 °C in 5 % CO_2 atmosphere for 24 h. The activity of the immobilized protein was accessed using the cell viability measurements as described in item 2.2.4. The One Way Anova test (Bonferroni´s Multiple Comparison Test) was used for all the statistical analysis and $p \leq 0,05$ values were considered statistically significant.

3. Results and Discussion

### 3.1 rhBMP2 purification and biological activity

Figure 1a shows the SDS-PAGE results for rhBMP-2 expression and identification. The firts line (kDa) shows the standard molecular weight proteins with 66, 45, 29, 20 and 14 kDa. Line 1 was empty, Lines 2 and 3 showed the protein fraction before (time zero) and after induction (16 h), respectively. At zero time point no protein was identified and after 16sec, a large band was identified in between 20 and 29 kDa. Lines 4 and 5 and shows the soluble and the insoluble fractions, respectively, in both of which the typical band of rhBMP-2 can be seen, indicating that the protein was identified in both the soluble and insoluble fractions.

Figura 1b shows the SDS-PAGE results for rhBMP-2 purification by column affinity chromatography using nickel resin equilibrated with buffer PBS 1x. Elution was performed with Imidazole in concentrations of 250mM and 500mM. kDa-molecular weight standard (66-45-29-20-14). Lane 1: Soluble fraction containing rhBMP-2 (pre-column). Lane 2: non bound proteins (Void). Lane 3: washing unbound proteins. Lanes 4 and 5: eluted fractions with Imidazole 250mM and 500mM respectively.

Figura 1c shows the Western Blotting of the purified rhBMP-2 fractions. Molecular Weight (kDa) (Precision Plus Protein TM Standards Dual Color, Bio-Rad). Lanes 1, 2 and 3: rhBMP-2 samples from purification by nickel column affinity chromatography ($\cong$ 24KDa).

The activity of rhBMP2 was tested in cell culture, following the procedure described in item 2.2.4., and the results are shown in Figure 1d. The growth of cell free of rhBMP2 was considered 100 % and used as the parameter to evaluate the growth of the cells with gradual increase of the protein. The cell's growth increased, increasing the amount of rhBMP2. For 0.15 µg, 0.3 µg and 0.5 µg well, the cells growth increased about 20 %, and, interestingly, for 150 µg/well the cells growth dramatically increased 60 % when compared to the control. These results indicated the biological activity of rhBMP2, in all the concentrations, but an optimum plateau for 150 µg/well.

Figure 1 - The schematic diagram of a and b SDS-PAGE results of Expression and purification of rhBMP2 respectively; c Immunodetection of rhBMP-2 using anti 6X-HisTag; d cell viability results using increasing amounts of rhBMP2 in C2C12 cells treated with purified rhBMP-2. Values are shown as percentage of cellular proliferation. Control: cells treated DMEM 0,5% FBS others groups are: cells treated with different concentrations of recombinant protein diluted in DMEM +0,5%FBS. * difference from the control ** between the control group and the treated.
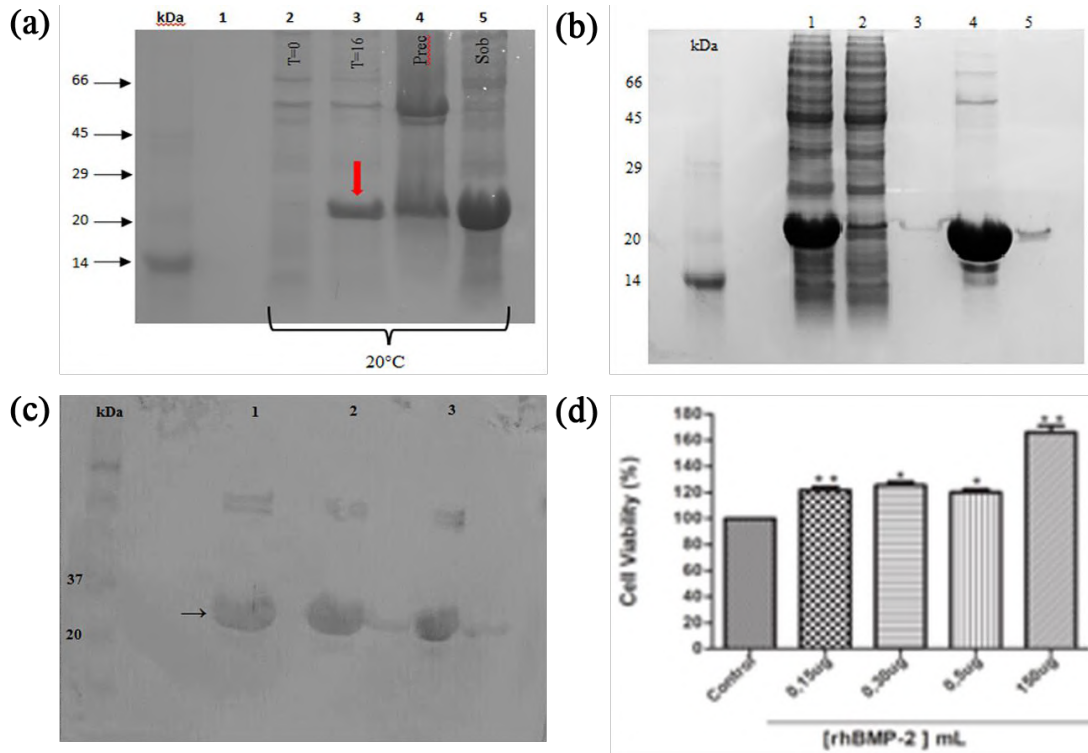
Source: Authors.

In Figure 1a, it is important to note that in addition to confirming the expression of the recombinant protein, especially on lane 5 of the SDS gel, we observed a good amount of rhBMP-2 in the soluble form. Most studies report the production of rhBMP-2 in the insoluble form, requiring the refolding process to make the protein biologically active, which could generate greater expenditure, in addition to lower product yield (Long et al., 2006; Retnoningrum et al., 2012; Sharapova et al., 2010; Zhang et al., 2011). However, in our work, we used a method in which we were able to express rhBMP-2 in soluble form, without the need for refolding, constituting an economical method of obtaining this protein with a satisfactory yield.

Figure 1b shows that partial purification of rhBMP-2 was efficient, eliminating most of the contaminating proteins (lane 4). It is noted that in the fraction represented by lane 5 by the Coomassie staining, there is no presence of contaminants. making this fraction perfect to be used in biological assays. To confirm the expression of rhBMP-2 we used the anti-HisTag antibody conjugated to alkaline phosphatase to make sure that we produced rhBMP-2 fused to six histidine residues. The immunodetection protocol in a semi-dry system was a success as we can see in figure 1c, as the antibody recognized rhBMP-2.

Figure 1d shows the biological activity of rhBMP-2 produced in soluble form and purified by affinity chromatography. This result was very important to validate the success of the bacterial production system used in this work. According to the graph, cell viability was higher in cells treated with rhBMP-2 at different concentrations compared to the control group (untreated cells).

3.2 Preparation of TEMPO-modified nanofibrillated cellulose (ToCNF) and its coupling to the maleimide moiety

The oxidation of the cellulose via TEMPO-mediated reaction was accessed by FTIR-ATR (shown in Figure 2a). The FTIR spectra of ToCNF display the typical peaks of cellulosic substrates at 1100 and 3300 cm-1 corresponding to C-O and O-H vibration bonds, respectively. The peak at 1600 cm-1 is assigned to the vibration of COO- groups, indicating the success of the oxidation, generating the binding sites for the coupling with FPAM (Saito et al., 2007). The content of carboxylic groups was 1.8 mmol.g-1, as determined by conductometric titration.

The coupling of the maleimide (FPAM) to ToCNF was carried out in two steps (Trovatti et al., 2017). First, the synthesis of the furan-protected amino maleimide (FPAM), followed by coupling to ToCNF. The FTIR spectrum of FPAM (Figure 3 a) shows bands at 1640, 3325 and 3380 cm-1 corresponding to the vibration of NH2, at 1694 and 1770 cm-1 corresponding to the C=O vibration of the maleimide ring, and bands in the region of 2800-2950 cm-1 corresponding to the vibrations of aliphatic CH bonds. It's grafting to ToCNF was indicated by the decrease in the intensity of the peak at 1600 cm-1 corresponding to the free COO-, the disappearance of the peak at 1638 cm-1 corresponding to free amine groups, and the appearance of the wide band at 1600-1640 cm-1, associated to the presence of the amide bond, suggesting the grafting had indeed occurred. After coupling, the ToCNF-FPAM was heated to release the furan ring from the structure by retro DA reaction, thus generating the maleimide moiety required for the reaction with the free sulfur atoms from the protein, as schematized in Figure 2b.

Figure 2 - FTIR-ATR spectra of ToCNF, FPAM and AM-ToCNF (a) and the scheme of the coupling of FPAM to ToCNF (b)
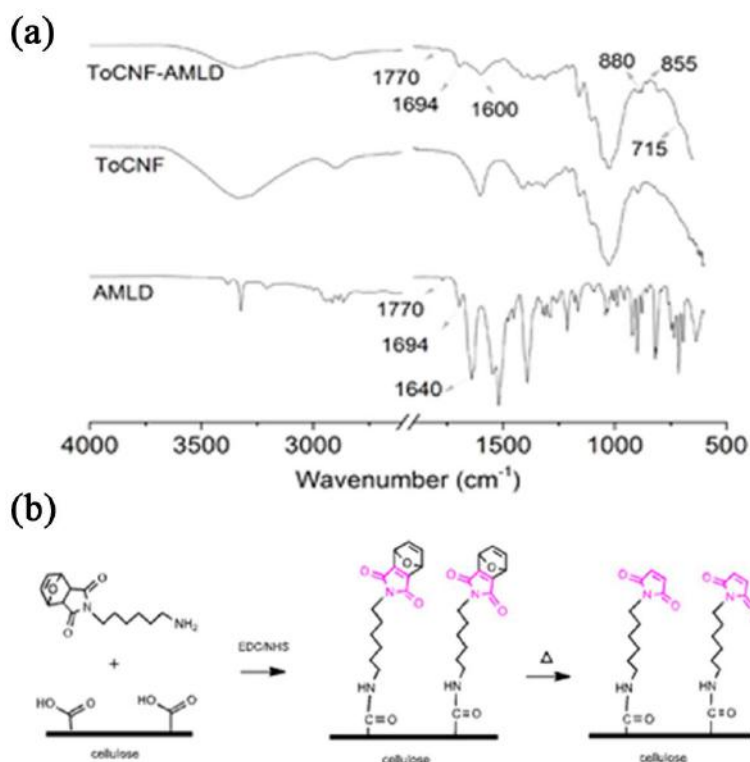
Source: Authors.

3.3 Grafting rhBMP-2 onto AM-ToCNF

Thiol-ene, as all the click chemistry reactions have desirable features such as high yield and no side products, specificity and no sensitivity to oxygen. Moreover, the UV-triggered thiol-ene reaction is fast and efficient. Among the thiol-ene reactions, maleimides can selectively target and crosslink free thiols in proteins, forming stable thioether linkages. The

reaction is used to develop stable protein conjugates (Kim et al., 2008).

Thus, the thiol-ene, namely the thiol-maleimide reaction, was strategically planned to apply the features of the click reaction in order to coupling rhBMP-2 to AM-ToCNF. The rhBMP-2 displays free thiol groups from the thioredoxin fused protein capable of reacting with the ene groups from the maleimide grafted at the surface of the ToCNF nanofibrils. The scheme of the chemical reaction is shown in Figure 3a and the FTIR results of the reaction are shown in Figure 3b. The grafting of the rhBMP2 to AM-ToCNF is evidenced by the set of changes in the rhBMP2/AM-ToCNF spectrum when compared to the AM-ToCNF spectrum.

The disappearance of the peaks at 623 and 917 cm-1, corresponding to the C=C bonds from the maleimide ring at the AM-ToCNF structure, strongly suggested that the grafting through thiol-ene reaction had indeed occurred. Thus, the disappearance of the double bond from the maleimide ring is the result of the grafting of the protein to the AM-ToCNF by thiol-ene reaction, leading to the formation of the C-S bond. In addition, the new peak at 1735 cm-1 is associated with the vibration of COOH groups from the protein side groups, the appearing of the peaks at 2919 and 2280 cm-1 assigned to the CH3 and CH2 also from side chain amino acids from the protein. The peak at 2900 cm-1 (ToCNF spectrum) is assigned to the CH2 stretching vibrations of cellulose (Trovatti et al., 2018). These results suggest the presence of the protein grafted to the ToCNF structure.

Figure 3 - (a) FTIR spectrum of AM-ToCNF (gray line) and rhBMP2/AM-ToCNF spectrum (black line) and the (b) schematic representation of the coupling reaction of BMP2 with ToCNF generating the ToCNF-BMP
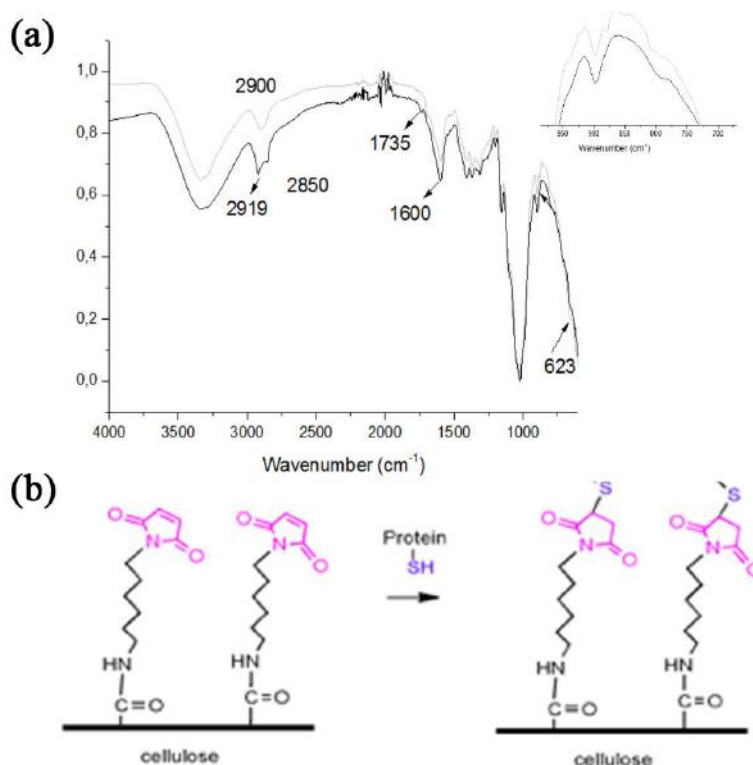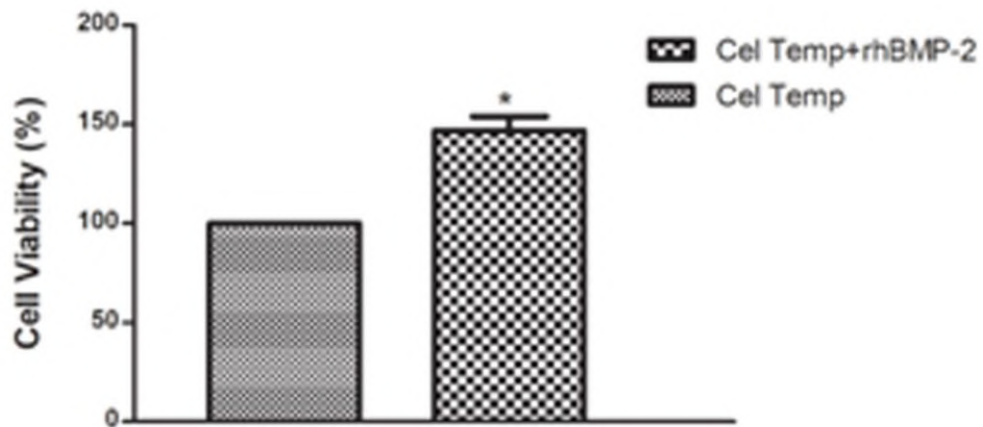

Source: Authors.

3.4 Cell proliferation at the rhBMP-2-ToCNF hydrogel

The cell proliferation assay shown in Figure 4 indicated the rhBBMP2-ToCNF substrate provided an increase of about 54 % on cell proliferation when compared to the ToCNF, free of BMP2, as substrate for cell growth. The result suggests the use of rhBBMP2 immobilized at the surface of polymers is capable of improve the cell grow, as well the fused protein

thioredoxin can be used to attach it at the substrates, using the thiol-ene photocalised strategy for coupling. This preliminary result represents a promising strategy for new advances in the field of grow factor grafting at the polymer surfaces for regenerative medicine.

Figure 4 - Cell proliferation and viability test (MTT) using ToCNF and rhBMP-2-ToCNF as substrate immobilized on the ToCNF surface.

Source: Authors.

4. Conclusion

The cloning of rhBMP fused to thioredoxin led to a high level of soluble synthesis of about 0,5 mg.mL-1 in its soluble form complexed to the thioredoxin protein. The attachment of thioredoxin to the surface of ToCNF hydrogel was performed using the specific reaction of thiol groups from the protein with the maleimide group from ToCNF nanofibers. Thioredoxin was used as the spacer for BMP protein, which activity was shown by increasing the rate of cells proliferation in vitro. These results showed for the first time the BMP-thioredoxin complex stimulated the cell growth without refolding and without releasing thioredoxin, increasing the perspectives of new studies about the use of the complex, besides the use of ToCNF as a carrier system for attachment of growth factors. Also, the results showed the activity of BMP coupled at the surface of ToCNF, as expected and in agreement with its mechanism of action, associated to the receptor at the membrane of the cells, showing it could not necessarily be free to exerted its activity, possibly because the coupling did not change is pristine conformation.

Acknowledgments

Declarations Conflict of interest

The authors declare that they have no conflict of interest.

References

Agrawal, V., & Sinha, M. (2017). A review on carrier systems for bone morphogenetic protein-2. *Journal of Biomedical Materials Research. Part B, Applied Biomaterials*, 105(4), 904–925. https://doi.org/10.1002/jbm.b.33599

Algar, W. R. (2017). A Brief Introduction to Traditional Bioconjugate Chemistry. Em Chemoselective and Bioorthogonal Ligation Reactions (p. 1–36). John Wiley & Sons, Ltd. https://doi.org/10.1002/9783527683451.ch1

Dimitriou, R., & Giannoudis, P. V. (2005). Discovery and development of BMPs. Injury, 36(3), S28–S33. https://doi.org/10.1016/j.injury.2005.07.031

Gautschi, O. P., Frey, S. P., & Zellweger, R. (2007). Bone Morphogenetic Proteins in Clinical Applications. *ANZ Journal of Surgery,* 77(8), 626–631. https://doi.org/10.1111/j.1445-2197.2007.04175.x

Karageorgiou, V., Meinel, L., Hofmann, S., Malhotra, A., Volloch, V., & Kaplan, D. (2004). Bone morphogenetic protein-2 decorated silk fibroin films induce osteogenic differentiation of human bone marrow stromal cells. *Journal of Biomedical Materials Research Part A*, 71A(3), 528–537. https://doi.org/10.1002/jbm.a.30186

Katagiri, T., Yamaguchi, A., Komaki, M., Abe, E., Takahashi, N., Ikeda, T., Rosen, V., Wozney, J. M., Fujisawa-Sehara, A., & Suda, T. (1994). Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. *Journal of Cell Biology*, 127(6), 1755–1766. https://doi.org/10.1083/jcb.127.6.1755

Khojasteh, A., Behnia, H., Naghdi, N., Esmaeelinejad, M., Alikhassy, Z., & Stevens, M. (2013). Effects of different growth factors and carriers on bone regeneration: A systematic review. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology*, 116(6), e405–e423. https://doi.org/10.1016/j.oooo.2012.01.044

Kim, Y., Ho, S. O., Gassman, N. R., Korlann, Y., Landorf, E. V., Collart, F. R., & Weiss, S. (2008). Efficient Site-Specific Labeling of Proteins via Cysteines. *Bioconjugate Chemistry,* 19(3), 786–791. https://doi.org/10.1021/bc7002499

Kumar, P., Nagarajan, A., & Uchil, P. D. (2018). Analysis of Cell Viability by the MTT Assay. *Cold Spring Harbor Protocols,* 2018(6). https://doi.org/10.1101/pdb.prot095505

LaVallie, E. R., DiBlasio, E. A., Kovacic, S., Grant, K. L., Schendel, P. F., & McCoy, J. M. (1993). A thioredoxin gene fusion expression system that circumvents inclusion body formation in the E. coli cytoplasm. *Bio/Technology (Nature Publishing Company),* 11(2), 187–193. https://doi.org/10.1038/nbt0293-187

Long, S., Truong, L., Bennett, K., Phillips, A., Wong-Staal, F., & Ma, H. (2006). Expression, purification, and renaturation of bone morphogenetic protein-2 from Escherichia coli. *Protein Expression and Purification*, 46(2), 374–378. https://doi.org/10.1016/j.pep.2005.09.025

Nandi, S. K., Roy, S., Mukherjee, P., Kundu, B., De, D. K., & Basu, D. (2010). Orthopaedic applications of bone graft & graft substitutes: A review. *The Indian Journal of Medical Research,* 132, 15–30.

Okamoto, M., Murai, J., Yoshikawa, H., & Tsumaki, N. (2006). Bone Morphogenetic Proteins in Bone Stimulate Osteoclasts and Osteoblasts During Bone Development. *Journal of Bone and Mineral Research,* 21(7), 1022–1033. https://doi.org/10.1359/jbmr.060411

Retnoningrum, D. S., Pramesti, H. T., Santika, P. Y., Valerius, O., Asjarie, S., & Suciati, T. (2012). Codon optimization for high level expression of human bone morphogenetic protein – 2 in Escherichia coli. *Protein Expression and Purification*, 84(2), 188–194. https://doi.org/10.1016/j.pep.2012.05.010

Ruhé, P. Q., Boerman, O. C., Russel, F. G. M., Mikos, A. G., Spauwen, P. H. M., & Jansen, J. A. (2006). In vivo release of rhBMP-2 loaded porous calcium phosphate cement pretreated with albumin. Journal of Materials Science: *Materials in Medicine,* 17(10), 919. https://doi.org/10.1007/s10856-006-0181-z

Saito, T., Kimura, S., Nishiyama, Y., & Isogai, A. (2007). Cellulose Nanofibers Prepared by TEMPO-Mediated Oxidation of Native *Cellulose. Biomacromolecules*, 8(8), 2485–2491. https://doi.org/10.1021/bm0703970

Sharapova, N. E., Kotnova, A. P., Galushkina, Z. M., Lavrova, N. V., Poletaeva, N. N., Tukhvatulin, A. E., Tukhvatullin, A. E., Semikhin, A. S., Gromov, A. V., Soboleva, L. A., Ershova, A. S., Zaĭtsev, V. V., Sergienko, O. V., Lunin, V. G., & Kariagina, A. S. (2010). [Production of the recombinant human bone morphogenetic protein-2 in Escherichia coli and testing of its biological activity in vitro and in vivo]. Molekuliarnaia Biologiia, 44(6), 1036–1044.

Termaat, M. F., Den Boer, F. C., Bakker, F. C., Patka, P., & Haarman, H. J. T. M. (2005). Bone morphogenetic proteins. Development and clinical efficacy in the treatment of fractures and bone defects. The Journal of Bone and Joint Surgery. *American Volume,* 87(6), 1367–1378. https://doi.org/10.2106/JBJS.D.02585

Trovatti, E., Cunha, A. G., Carvalho, A. J. F., & Gandini, A. (2017). Furan-modified natural rubber: A substrate for its reversible crosslinking and for clicking it onto nanocellulose. *International Journal of Biological Macromolecules,* 95, 762–768. https://doi.org/10.1016/j.ijbiomac.2016.11.102

Trovatti, E., Tang, H., Hajian, A., Meng, Q., Gandini, A., Berglund, L. A., & Zhou, Q. (2018). Enhancing strength and toughness of cellulose nanofibril network structures with an adhesive peptide. Carbohydrate Polymers, 181, 256–263. https://doi.org/10.1016/j.carbpol.2017.10.073

Wang, R. N., Green, J., Wang, Z., Deng, Y., Qiao, M., Peabody, M., Zhang, Q., Ye, J., Yan, Z., Denduluri, S., Idowu, O., Li, M., Shen, C., Hu, A., Haydon, R. C., Kang, R., Mok, J., Lee, M. J., Luu, H. L., & Shi, L. L. (2014). Bone Morphogenetic Protein (BMP) signaling in development and human diseases. *Genes & Diseases,* 1(1), 87–105. https://doi.org/10.1016/j.gendis.2014.07.005

Xiao, Y.-T., Xiang, L.-X., & Shao, J.-Z. (2007). Bone morphogenetic protein. *Biochemical and Biophysical Research Communications,* 362(3), 550–553. https://doi.org/10.1016/j.bbrc.2007.08.045

Yu, N. Y. C., Schindeler, A., Peacock, L., Mikulec, K., Baldock, P. A., Ruys, A. J., & Little, D. G. (2010). In vivo local co-delivery of recombinant human bone morphogenetic protein-7 and pamidronate via poly-D, L-lactic acid. *European Cells & Materials,* 20, 431–441; discussion 441-442. https://doi.org/10.22203/ecm.v020a35

Zhang, Y., Ma, Y., Yang, M., Min, S., Yao, J., & Zhu, L. (2011). Expression, purification, and refolding of a recombinant human bone morphogenetic protein 2 in vitro. *Protein Expression and Purification*, 75(2), 155–160. https://doi.org/10.1016/j.pep.2010.07.014