Mammalian Cell Culture on a Novel Chitosan-Based Biomaterial Crosslinked with Gluteraldehyde

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Summary: A novel (Chitosan-*g*-Glycidyl Methacrylate)-Xanthan, (CTS-*g*-GMA)-X, hydrogel was successfully synthesized at Cinvestav Queretaro. In the present work, we attempt to study the in vitro biocompatibility of (CTS-*g*-GMA)-X hydrogels as scaffolds for neural cells obtained from cerebral cortex of mice; skin cells from skin of mice; and chondrocytes from human cartilage. The results obtained in the live/dead assay show that the materials maintain the morphology and viability of the cells. However the high friability of the materials prevented an extended test time in this assay. Therefore, glutaraldehyde (GL) was added to the structure as crosslinking agent to enhance friability. All results point out that [(CTS-g-GMA)-X]-GL hydrogel is viable as a scaffold for chondrocytes and skin cells; and (CTS-g-GMA)-X for neural cell due to their physicochemical properties and biocompatibility with the cells for both materials.

Keywords: cell culture; chitosan; crosslinking; tissues engineering; xanthan

Introduction

Tissues engineering is the integration of multidisciplinary science, included fundamental principles from material science and molecular biology in efforts to develop biological substitutes for tissues and organs.[1] In its simplest form a tissues engineering scaffold provides mechanical support as a synthetic extracellular matrix to organize cells into a three dimensional structure, shape, and cell-scale architecture for neo-tissue construction in vitro or in vivo as seeded cells expand and organize. Depending on the tissue of interest and the specific application the required scaffold material and its properties will be quite different. Most the biomaterials used to date comprise a class of synthetic polyesters such as poly (L-lactic acid) (PLLA) and poly (L-glycolic acid), and/or biological polymers such as alginate, chitosan, collagen and fibrin.[2,3]

A biopolymer of growing interest for tissues engineering is chitin, the second most abundant polymer in the earth, which can find in important quantities in exoskeleton of crustaceans, insects, and cellular walls of some fungi.[4] Chitin is a copolymer with N-acetyl-glucosamine and N-glucosamine units randomly or block distributed throughout the biopolymer chain depending on the processing method used to derive the biopolymer. Chitosan is a polysaccharide consisting of $\beta(1–4)$ linked D-glucosamine residues with a variable number of randomly located

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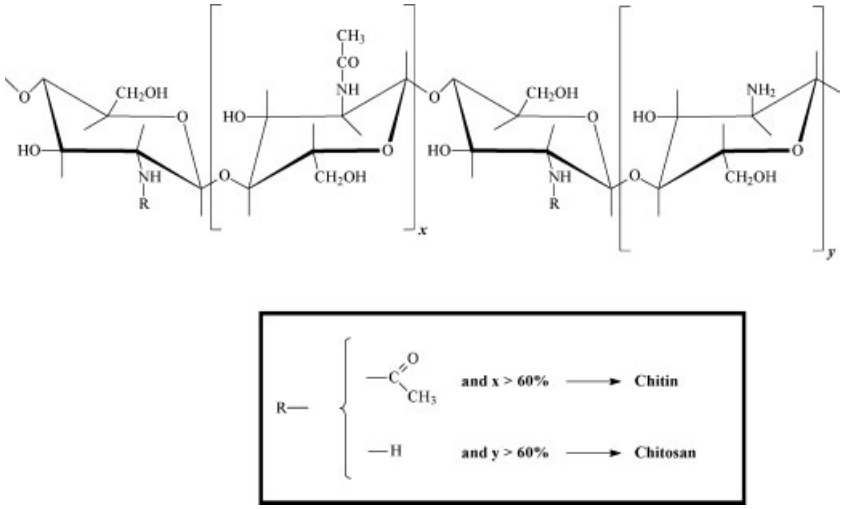

Figure 1.
Schematic representation of the chitin and chitosan depicting the co-polymer character of the biopolymers.[4]

N-acetyl-glucosamine groups, with a semi crystalline structure.[4,5]

The frontier between Chitin/Chitosan can be located in the percentage of N-acetyl-glucosamine units, Figure 1, if this is greater of 60% the polymer is called Chitin; otherwise, if the percentage is less of 40%, the polymer is called Chitosan. Both polymers,[6,7,8] Chitin and Chitosan, belong to glycosaminoglycans (GAG) family and exhibits numerous interesting biological and physico-chemical properties; both biopolymers has been researched due to their solubility in dilute acids, being Chitosan more reactive and avoiding different chemical reactions with the aim of produce new materials for several applications such as biotechnology.[9,10]

Chitosan is a cationic and basic polyelectrolyte, for this reason is dissolves in aqueous acidic media forming viscous solutions that can form a hydrogels by crosslinking reaccions, which occurs between a structural unit of chitosan chain and a structural unit of a polymeric chain of another type;[11,12] these materials has been used by many investigations groups in the world like Berthod *et. al*. that developed a sponge collagen chitosan for skin reconstruction obtaining artificial skin similar to normal dermis; also Artphop Neamnark and his group found that chitosan fibres added with hexanoyl by the electrospinning technique that could be accepted as a material for keratinocytes growth.[13,14]

The International Cartilage Repair Society (ICRS) at 2007 evaluted some materials of chitosan and glicerol phosphate in the treatment of cartilage repair and observed that the implants favored intermembranous bone formations;[15] also Yamane used chitosan conjugated with hyaluronan to obtain a biomimetic matrix for chondrocyte adhesion an proliferation.[13] Additional Y. L. Lin *et al*. obtained a chitosan matrix wtih gold nanoparticles to facilitate nerve cell proliferation and growth, however the material affects the cellular response[16] this fact is important because continue with the study about chitosan as biomaterial and its possible biocompatibility with differents tissues.

In the present work, we attempt to study the in vitro biocompatibility of chitosan based hydrogels as scaffolds for different type of cells such as chondrocytes, neurons, skin cells and enteric nervous system cells evaluated means microscopy images.

Materials and Methods

Synthesis Methods

Chitosan-g-Glycidyl Methacrylate (CTS-g-GMA) Synthesis

This material was synthesized in stoichiometric molar ratios CTS:GMA of 1:1, a diagram of the chemical reaction is shown in Figure 2. The solution was prepared mixing a known chitosan quantity in a 0.4 M acetic acid solution; this material was poured in a flask and then 0.05 M 15% KOH (w/w, respect to the total reactive mass) was added. The GMA was added slowly to obtain the correct stoichiometric molar ratio. The resulting mixture, which had a pH of about 3.8 was enclosed, its temperature was raised to $60 \pm 1\,^{\circ}C$, bubbled with nitrogen gas and then maintained with a constant magnetic agitation for an optimum reaction time of 2 hours. The product was cooled in an ice bath for 15 minutes to complete the reaction. The product had a milky appearance that in the presence of acetonitrile precipitated as a white solid. The minimum ratio necessary to observe precipitation was nearly 8:1 (V:V, acetonitrile:product). This reaction was discussed in a previous work.[17]

Xanthan Solution (X)

The solution was prepared dissolving xanthan gum in distilled water by means of magnetic agitation.

Chitosan-g-Glycidyl Methacrylate- Xanthan Hydrogel Synthesis ((CTS-g-GMA)-X)

In an Erlenmeyer flask a known quantity of CTS-*g*-GMA was placed in dry weight, and distilled water was added (pH mixture 6.8). When the CTS-g-GMA was dispersed homogeneously, the xanthan solution was added slowly, the vial was closed and then the nitrogen flow was introduced, the temperature was kept at $50 \pm 1\,^{\circ}C$, and constant magnetic agitation was provided, the scheme of the reaction is showed in Figure 3. After four hours, the optimum reaction time, the gas flow, heating and agitation were stopped. The product was cooled in an ice bath for 15 minutes in order to finish the reaction. The pearls were washed once with distilled water in order to eliminate the residual reactants. The product was dried in air and then in an oven for one hour at $45\,^{\circ}C$.[18]

Chitosan-g-Glycidyl Methacrylate-Xanthan-Glutaraldehyde Hydrogel Synthesis ([(CTS-g-GMA)-X]-GL)

A solution of CTS-g-GMA was prepared in acetic acid 0.4 M. Separately xanthan was solved in distilled water until obtain a homogeneous solution, after this glutaraldehyde (GL) was added (7% respect reaction total weight). Once that both solutions were homogeneous, the X-GL solution was added slowly, the reaction temperature was kept at $40 \pm 1\,^{\circ}C$ with constant magnetic agitation. After two

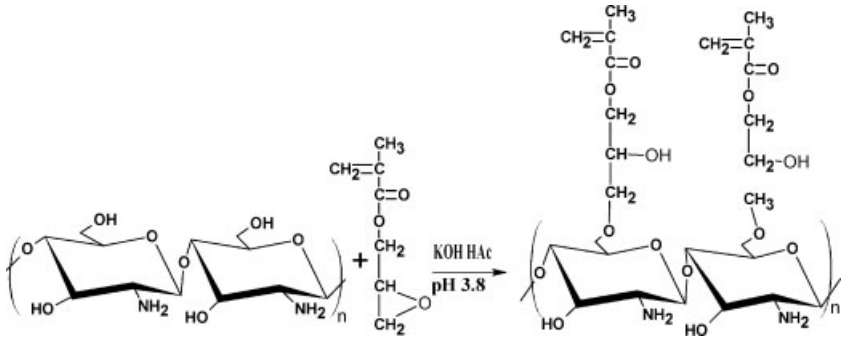

Figure 2.
Proposed mechanism of the chemical reaction CTS-*g*-GMA as described elsewhere Flores-Ramirez et al., 2005.[17]

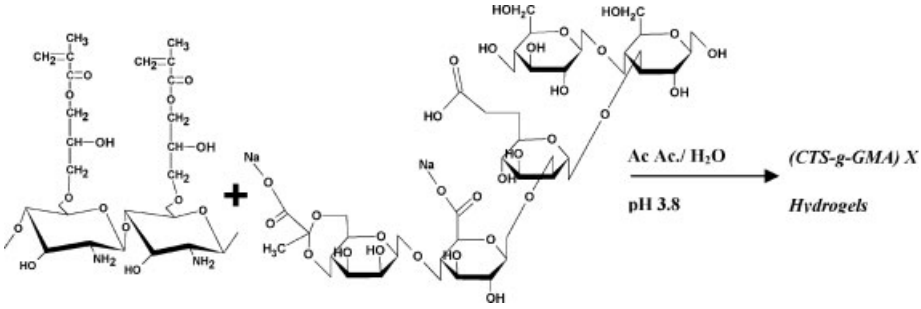

Figure 3.
Proposed mechanism of the chemical reaction of the CTS-*g*-GMA with Xanthan. The resulting structure is not fully elucidated due to the complex interactions between anionic polymers Chitosan and Xanthan gum.[18]

hours the reaction was stopped cooling the flask in an ice bath for 15 minutes. /the product was recovered and dried in an oven overnight at 50 °C for subsequent characterization and applications.

Cell Culture

Cell Culture onto the Biomaterials

Polymer discs, with GL and without GL, were placed in multi-well plates for cell culture. The pH of the polymers was adjusted rinsed discs, first with phosphate buffered saline (PBS) and after with fetal bovine serum and DMEM-F12 media; adding 100 μL each one, twice every 2 minutes. After reach the optimum pH the cells were added.

Skin Cells Culture

All experiments were performed according to the protocol approved by the Animal Care and Use Committee of The National Autonomous University of Mexico (UNAM) and National Rehabilitation Institute (INR).

Full-thickness skin tissue was obtained from Balb/c mice and digested in PBS (Gibco, Life Technologies, Gaithersburg, MA) containing 0.25% trypsin (Gibco, Canada). Samples were incubated three hours and cellular debris was removed. Cells were maintained in culture media, Dulbecco's modified Eagle's medium, (DMEM), supplemented with 10% (v/v) of fetal bovine serum, 100 U/mL penicillin; 100 mg/L streptomycin and 0.25 μg/mL amphotericin B in 25 cm^2 tissue flasks (Corning® HTS Transwell®), and cultured at 37 °C, 90% humity and 5% CO_2; until confluent monolayer was obtain. At primary culture, cells were tripsinized and seeded onto the polymer (CTS-*g*-GMA)-X and [(CTS-*g*-GMA)-X]-GL, then were transferred to a multi-well plate and incubated for 24 hrs.[19,20]

Neural Cells Culture

Cerebral cortices were dissected from Balb/C mice and incubated in 0.2% of collagenase I (Worthington, Freehold, NJ) for one hour. The cells were dispersed by mechanical disaggregation using progressively narrow fire polished, reduce-bore Pasteur pipettes. Samples were filtered using nylon meshes with pore of 100 μm. Cell pellet was re-suspended in media culture DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin; 100 mg/L streptomycin and 0.25 μg/mL amphotericin B (Gibco, Life Technologies), seeded in 25 cm^2 tissue flasks and incubated at 37 °C, 90% humidity and 5% CO_2.[21–23] After 17 days of culture cells hardvested and seeded onto the (CTS-*g*-GMA)-X and (CTS-*g*-GMA)-X-GL polymers and transfer to a multi-well plate (Corning® HTS Transwell®, Sigma – Aldrich, St. Louis Missouri) and cultured for 24 hrs.[19,20]

Chondrocytes Culture

Chondrocytes from normal human cartilage with informed consent and local ethical committee approval were isolated by mechanical and enzimatically collagenase

digestion (0.3% collagenase II Worthington, Freehold, NJ) for 4 hours. Cells were maintained in culture media DMEM-F12 supplemented with 10% (v/v) of fetal bovine serum, 100 U/mL penicillin; 100 mg/L streptomycin and 0.25 μg/mL amphotericin B (Gibco, Life Technologies) in 25 cm^2 tissue flasks and cultured at 37 °C, 90% humidity and 5% CO_2; until confluent monolayer was obtained. Cell at first passage were trypsinized (Gibco, Canada), and cells re-suspended in supplemented culture media, 2×10^6 cells/mL were seeded onto the (CTS-g-GMA)-X and [(CTS-g-GMA)-X]-GL polymers, then were cultured in a multi-well plate (Corning® HTS Transwell®, Sigma – Aldrich, St. Louis Missouri) for 24 hrs.

Enteric Nervous System (ENS) cells culture

ENS cells were isolated from neonate mice, the small intestine was extracted and washed with a Hanks balanced salted solution (HBSS, Gibco, Life Technologies) with 1% (v/v) of antibiotic (100 U/mL penicillin; 100 mg/L streptomycin and 0.25 μg/mL amphotericin B, Gibco, Life Technologies). Once fragmented it move to a tube for digestion with dispase type I (0.1 mg/mL; Boehringer Ingelheim, Ingelheim am Rhein, Germany), colagenase type IX raw extract (300 U/mL; Sigma Chemical Corp., St. Louis) and DNAase type I (10 mg/mL; Boehringer Ingelheim, Ingelheim am Rhein, Germany). Later is done, other mechanical dispersion, was done using pipette tips. After that samples were centrifuged and the supernatant was extracted taking care of the pellet which was resuspended in 1 ml of cell culture media DMEM (Gibco, Life Technologies). Finally the pellet was filtered through a 100 μm cell strainer (Falcon, Bedfors, MA) and cultured in T-25 flasks with DMEM supplemented with 10% (v/v) of fetal bovine serum, 100 U/mL penicillin; 100 mg/L streptomycin and 0.25 μg/ml amphotericin B (Gibco, Life Technologies), bFGF y EGF (20 ng/mL) at 37 °C with atmosphere of 5% CO_2. When the cells had an optimum confluence were tripsinized and cultured onto the polymers in a cell density of 2×10^6 cells/mL and were cultured in a multi-well plate (Corning® HTS Transwell®, Sigma – Aldrich, St. Louis Missouri) for 24 hrs.

Cell Viability

In order to evaluate cell viability a LIVE-DEAD® Viability/Cytotoxicity Kit (L-3224, Molecular Probes, Invitrogen) was used after 24 hrs of each cell culture onto every polymer performed according to the manufacturer's instructions. Cell cultures were then observed by confocal microscopy LSM510 – META at $\lambda = 500$–530 nm to green (viable cell) and $\lambda = 650$–710 to red (dead cells).

SEM

The culture media was removed of each well and the cells was fixed with a solution of 4% (v/v) paraformaldehyde (Sigma – Aldrich, St. Louis Missouri) in PBS (Gibco, Life Technologies) for 32 hours, later this the samples were analyzed in a environmental scanning electron microscope PHILLIPS model XL-30 ESEM.

Results

Infrared Analysis

Figure 4 shows the FTIR spectra from pure chitosan, xanthan, and CTS-*g*-GMA. The spectra for chitosan and CTS-*g*-GMA are very similar to each other except for the shift of the NH_2 peak (1597 cm^{-1} for chitosan and 1580 cm^{-1} for CTS-*g*-GMA). The decrease in wavenumber could be associated to a protonation of the NH_2 group (larger effective mass) induced by the acid environment in the treatment described in the section devoted to the CTS-*g*-GMA synthesis. Since the intensity of that peak was kept approximately constant, we concluded that the functionalization of the chitosan by the GMA was not through the amine group. This suggests that the addition of the GMA was done through the meridional–OH group, which is the most reactive site in acid environment.[17,18]

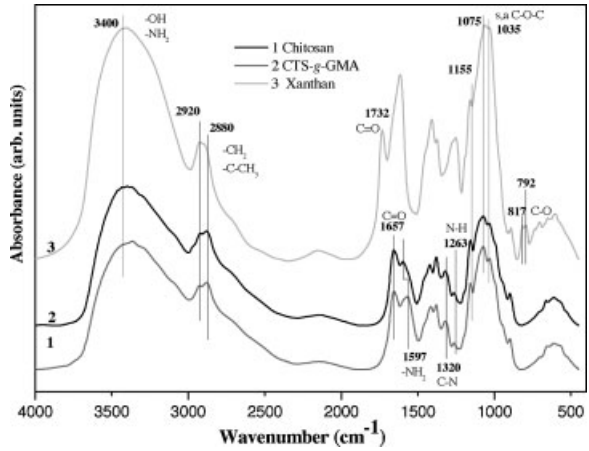

Figure 4.
Spectra of precursor polymers Chitosan, CTS-g-GMA, and Xanthan; show representative bands of them.

The origin of most of the peaks shown in Figure 4, for xanthan, has been reported elsewhere[24–26] except for the doublet at 817 and 792 cm^{-1}. Those could be a weak contribution due to the C–O link of the ring in the cyclic ether. The position suggests an ether molecule in the cis-position. In addition these bands could contribute to the swinging vibrations of the methylene groups from the cyclic ether.

Figure 5 shows the infrared spectra for the CTSGMA-X hydrogel, glutaraldehyde, and [(CTS-g-GMA)-X]-GL; most of the peaks are discussed in the previous paragraphs. It was not possible to observe, in (CTS-g-GMA)-X hydrogel, the chitosan characteristic (C=O and $-NH_2$ at 1657 and 1597 cm^{-1}) bands. The band in 1409 cm^{-1} is mainly the symmetric stretching of the carboxylate group in the xanthan, although it has contributions of bending vibrations of the hydroxyl groups in chitosan and xanthan. The absorption near the 1265 cm^{-1} band corresponds to the stretching vibrations of the C–O group of the ether and ester groups. Finally, as an complementary

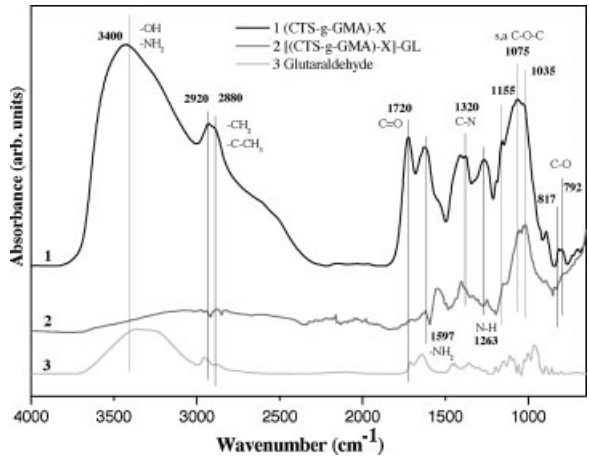

Figure 5.
Representative spectra for the hydrogels used as scaffolds for cell culture.

information in all the spectra the doublet with absorptions in 815 and 798 cm^{-1} are associated to the vibrations in the cyclic ether ring and for the swinging mode of the methylene group out of the heterocyclic ring in xanthan.

The difference among these systems could be due to the effect of the chemical bonding between the OH́s and the carboxylate groups. The latter observations and our previous discussion support the idea of a strong link between the xanthan and the CTS-*g*-GMA.[17,32–34] In addition, the presence of GL in the structure of the [(CTS-g-GMA)-X]-GL promote several shifts in characteristic bands such as 2920–2880 for CH_2 and CH_3, and around 1597 assigned to NH_2 due to the interaction between GL, and xanthan with the chitosan's amine groups. This fact confirms the addition of GL which provides strength to material for their application in the cell culture.

Cell Culture

The (CTS-*g*-GMA)-X hydrogel presents adequate properties of swell for cell culture, however due to the media pH 7.4, and the chemical properties of xanthan gum the polymer begin to disaggregate decreasing their mechanical properties. This fact

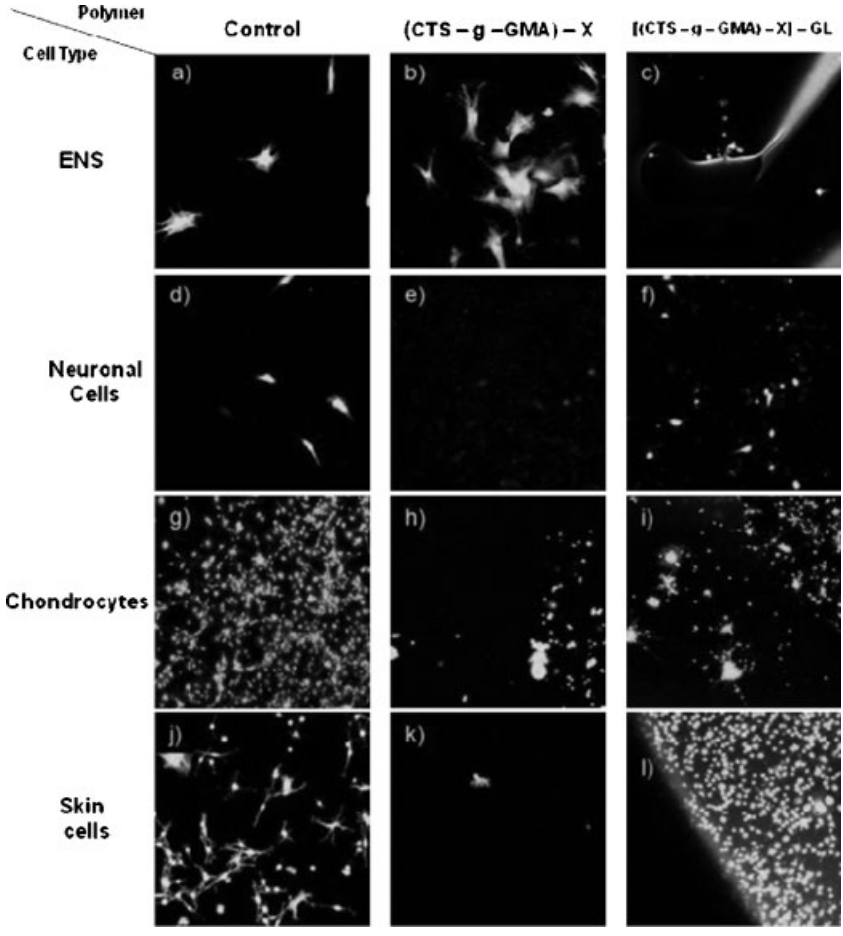

Figure 6.
Morphology and cell viability after 24 hours of culture onto each polymer. Cells control with polystyrene (a, d, g and j). Cell culture in (CTS-g-GMA)-X (b, e, h and k). Cell culture in [(CTS-g-GMA)-X]-GL (c, f, i and l), Living cells were stained with Calcein (green). All culture was observed under a confocal microscope (x 10).

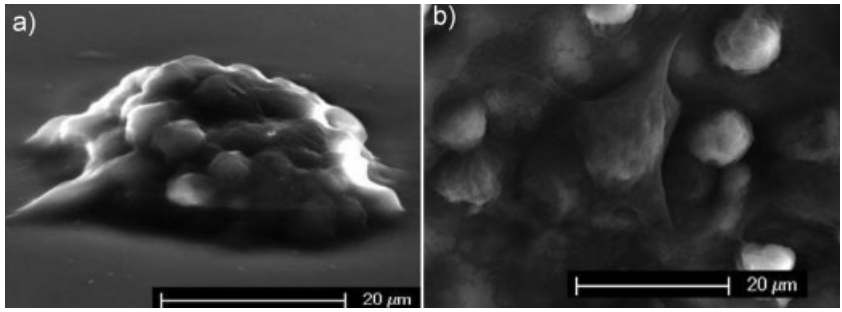

Figure 7.
ESEM analysis of biopolymer seeded with two different cell types. [(CTS-g-GMA)-X]-GL seeded with (a) images of chondrocytes isolated from human cartilage at second passage seeded onto [(CTS-g-GMA)-X]-GL, (1500X) cells were isolated from human cartilage (x 1500) .They remained round and were still attached to the polymer; (b) Polymer seeded and cultured for 24 hrs with ketarinocytes isolated from mice skin and cultured at first passage we can distinguished the nucleus and cytoplasm (1200X) and also attached to the polymer.

promotes the chemical modification with glutaraldehyde to enhance the mechanical properties and reach a better stability at pH 7.4.

Results showed that the material (CTS-g-GMA)-X, due to its mechanical properties, provides an appropriate environment for cell survival ESN and neuronal cell at 24 hours of culture (Figures 6b and 6e); case contrary to these cell types grown on [(CTS-g-GMA)-X]-GL, in which a greater degree of cross-linking due to the presence of glutaraldehyde, modifies the swelling capacity providing greater rigidity to the material thus it does not extracellular matrix emulates conditions for ESN and neuronal cell (Figures 6c and 6f). On the other hand, a lower capacity of swelling [(CTS-g-GMA)-X]-GL (Figures 6i and 6l) provides an area where both chondrocytes and skin cell had a higher cell viability (CTS-g-GMA)-X (Figures 6h and 6k), likewise, these cell types did not lose their typical morphology which is possible to confirm in the ESEM after 24 hours of culture (Figure 7). Nevertheless, both materials maintain cells viability, are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually non-fluorescent cell-permeate Calcein-AM to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells in contrast EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells. EthD-1 is excluded by the intact plasma membrane of live cells, the different type of cells compared with polystyrene, material of reference, where the viability and morphology remain 24 hours after the cell culture. All this results promote to [(CTS-g-GMA)-X]-GL hydrogel like a scaffold for chondrocytes and skin cells; and (CTS-g-GMA)-X for cells type neurons; due to their mechanical properties and biocompatibility with the cells for both materials.[35] But still remains to verify that non-submission process of dedifferentiation for skin, chondrocytes and neuronal cells, and not for the case of cells in the ESN as this type of cells could differentiate until neurons agreement Suárez-Rodríguez and Belkind-Gerson (2004).[36]

Conclusions

Non-crosslinked biomaterials have proven suitable for *In vitro* ENS cultures. The crosslinked one provides a good scaffold for in vitro SK and CT cultures. This study

shows that chitosan-based biomaterials can be chemically fine-tuned to mimic ECM for cell growth and proliferation. The next step is to conduct verification of some specific cellular markers to demonstrate cell phenotype and not only cell morphology and viability.

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