Structural Effect of Different EDC Crosslinker Concentration in Gelatin-Hyaluronic Acid Scaffolds

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Abstract

Introduction: Gelatin and hyaluronic acid are two biopolymers with different applications in tissue engineering. They may be employed to construct diverse scaffolds that allow cells to differentiate and proliferate on them. In order to obtain the best functional and mechanical conditions in scaffolds, they must be crosslinked to form covalent links between gelatin and hyaluronic acid. The crosslinker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) is a compound widely used due to its low cytotoxicity. Besides, the concentration of the crosslinker may modify the physical properties and morphological characteristics of scaffolds when it forms covalent links between biopolymers, helping to construct different kinds of scaffolds used for developing soft tissues. However, the development of scaffolds made of gelatin and hyaluronic acid crosslinked with EDC has been poorly studied. In addition, the concentrations used for crosslinking gelatin and hyaluronic acid are contradictory. Therefore, the aim of this study was to analyze the structure of gelatin/hyaluronic acid scaffolds crosslinked with EDC.

Methods: Gelatin-hyaluronic acid scaffolds were prepared by direct freeze-drying. Afterwards, They were crosslinked with different concentrations of EDC (6, 30, 50 and 60 mM) for 12 h.

Results: This research has demonstrated that the gelatin/hyaluronic acid scaffolds crosslinked with the highest concentrations of the crosslinker had fewer water concentration absorbed, pore size diminished and pore number increased in comparison with control groups. Despite scaffolds composition has not changed in any of the concentrations, the bone marrow mesenchymal cells mortality percentage increased when cells were placed on the scaffolds of concentration 60 mM, perhaps for the residual 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride found in the scaffolds.

Conclusion: Our results revealed that different EDC concentrations may modify the physical and biological characteristics of gelatin/hyaluronic acid scaffolds; as a result, the scaffolds obtained may be used for the manufacture of different tissues in regenerative medicine.

Keywords: Biomaterials; EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride); Gelatin; Hyaluronic acid; Tissue engineering

Introduction

The connective tissue is composed of cells and extracellular matrix (ECM). This one, among other characteristics, it owns the capacity of giving support to the cells through binding motifs recognized by integrins; this tissue is insoluble and it does not have the capacity of diffusion. The ECM is formed by macromolecular nets highly hydrated composed by glycoproteins like collagen, elastin, fibronectin and laminin; and glycosaminoglycans like hyaluronic acid; chondroitin 6-sulfate, dermatan sulfate and hepan sulfate, which have been employed for constructing diverse tridimensional scaffolds very similar to the extracellular matrix from the connective tissue [1,2].

Collagen type I is the principal component of ECM. It has been used for constructing tridimensional structures, treating burn lesions and skin ulcers of different origin [3-6]. However, there are other molecules very useful for producing scaffolds. Gelatin (Ge) is a product derived from collagen. It is easy to extract, soluble in water and is completely reabsorbed in vivo. In addition, it possess low immunogenicity and due to its physicochemical properties like the presence of functional groups such as arginine, aspartic acid, glycine, proline and other aminoacids, it allows the attachment of cells such as fibroblasts [7,8]. Furthermore, the adhesion properties of Ge are regulated for the aminoacid sequence RGD (arginine-glycine-aspartic acid), that may be recognized for many integrins in cells from connective tissue [9-11]. Besides, it has been demonstrated that the cellular functions in tridimensional scaffolds are better when natural proteins are used, instead short aminoacid sequences [12]. In addition, Ge has a triple helix structure equivalent such as collagen, that may form essential links for constructing tridimensional nets [13]. As a result, the Ge properties make this molecule an excellent candidate for drug delivery and for being used in tissue engineering [14-16].

On the other hand, the hyaluronic acid (HA) is a long chain polysaccharide, biocompatible and biodegradable, with high capacity to retain water under biological conditions [17]. HA is formed for repetitive disaccharide units of D-glucuronic acid and N-acetyl-D glucosamine, which are extensively found in ECM [18]. It has been shown that HA stabilizes and organizes the ECM, due to its interactions with other proteins, and also, regulates the adhesion and proliferation.
of fibroblasts by the receptor CD44 [19]. HA biomedical tissue engineering includes the construction of scaffolds for wound healing and repairing bone defects [20,21], treatment against arthritis and as a component of implant materials [22].

In order to use a macromolecule to construct a biocompatible scaffold it should be crosslinked. Crosslinking improves the functional characteristics and mechanical structure of biomaterials used for tissue engineering. In this process a covalent crosslinking between two or more macromolecules occurs when the material is treated with different crosslinking agents [23].

The crosslinker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) unlike other molecules like glutaraldehyde, is not part of the final product after the crosslinking reaction with the molecules that conforms the scaffold, so it does not induce cytotoxic effects. EDC reacts with the biopolymers hydroxyl groups in order to form active O-urea, which reacts with amino groups creating an amide link and releasing isourea. This last molecule is a sub product soluble in water that may be discharged easily [18]. To the best of our knowledge, EDC has been used as crosslinker of scaffolds made of collagen and calcium phosphate [24] and also in corneal replacement or regeneration, where collagen, gelatin and hyaluronic acid are the most common molecules used [25]. Nevertheless, the concentrations of the crosslinker used in scaffolds made of Ge and HA are contradictory. So, the aim of this investigation is the characterization of the chemical and morphological properties of Ge/HA scaffolds crosslinked with different concentrations of EDC.

Material and Methods

Animals

Six 250 g Wistar male rats were used for each experiment. Animals were kept under pyrogen free condition, and fed ad libitum in the animal facilities of the Department of Cell and Tissue Biology from the Faculty of Medicine, UNAM. This investigation was approved by the Faculty of Medicine Ethical Board, UNAM and Animal Sacrifice was realized according the Mexican Official Norm NOM 062-ZOO-1999.

Reactives

Type B Ge obtained from bovine skin (Sigma-Aldrich, St. Louis, MO, USA), with a molecular weight of 20,000-25,000 Da, with a gel-strength in the Bloom scale of 225 g and an isolectric point (IP) of 4.7-5.2. HA obtained of Estreptococcus equi (Sigma-Aldrich, St. Louis, MO, USA), with a molecular weight of 20,000-25,000 Da, with a gel-strength in the Bloom scale of 225 g and an isolectric point (IP) of 4.7-5.2. HA obtained of Estreptococcus equi (Sigma-Aldrich, St. Louis, MO, USA), with a molecular weight of 403.3 g/mol, soluble in water (5 mg/ml) and with 99% purity. EDC, with a molecular weight of 191.7 g/mol and water solubility of < 100 mg/ml, was obtained from Sigma-Aldrich, St. Louis, MO, USA) with a molecular weight of 403.3 g/mol, soluble in water (5 mg/ml) and with 99% purity. EDC, with a molecular weight of 191.7 g/mol and water solubility of < 100 mg/ml, was obtained from Sigma-Aldrich, St. Louis, MO, USA).

Ge/HA scaffolds manufacturing

Ge and HA were dissolved in a 4:1 proportion respectively in distilled water with constant agitation for 30 min at 50 °C. One ml of Ge/HA solution were frozen in liquid nitrogen in order to be lyophilized for 12 h at -56 °C with 0.036 m Bar of atmospheric pressure. Afterwards, the scaffolds were treated for 12 h at 4 °C with different concentrations of EDC (6, 30, 50 or 60 mM) dissolved in ethanol 96%. Subsequently, the scaffolds were washed three times with distillate water and lyophilized under the conditions explained above. Scaffolds were incised in discs of 9mm diameter and 3 mm width. Subsequently, these samples were used to perform biological and physical tests.

Pores quantification

Scaffolds crosslinked with different EDC concentrations were processed in order to obtain 5 µm histological sections, which were stained with hematoxylin and eosin (H-E). For histological evaluation three samples of each condition were analyzed, 10 areas were randomly selected and the number of pores was evaluated at 1000X in a Nikon E801 microscope. The average of the pore number in the scaffolds was expressed as number of pores/mm².

Scanning electron microscopy (SEM)

The scaffolds crosslinked with different concentrations of EDC were coated for 1 min with gold in an ionizator Polaron model 11HD. After wards, the scaffolds were examined with a ZEISS DSM950 Scanning Electron Microscope. Four photographs were taken per each scaffold at 1500X. The images obtained were analyzed with the Image-Pro Plus 7.0 program (Media Cybernetics, Inc. San Diego CA) in order to measure the pore size.

Microanalysis energy dispersive X-ray spectroscopy (EDS)

In order to identify the elemental chemical composition of the scaffolds, they were analyzed with the EDS technique with the Noran probe, Voyager model 4.2.3., coupled in a Scanning Electron Microscope JEOL JSM6060LV model. From each sample, 10 random fields were selected and elements concentration analyzed.

Absorption capacity

Crosslinked scaffolds with 9 mm diameter by 3 mm width were weighed and immersed in 1 M phosphate buffer saline for 1 h at room temperature. Exceeding water was removed from the scaffolds by placed them on absorbent paper for 2 s, subsequently they were reweighed. Weight in grams, corresponding to the absorption capacity, was obtained with the following formula: CA = PH—PS, where CA is the absorption capacity, PH is the wet weight of the scaffold and PS is the dry weight of the scaffold. All experiments were performed in triplicate.

Cytotoxicity and cell culture in vitro

Ge/HA scaffolds were sterilized by immersing in 70% alcohol and placing at UV light all night, also they were washed 3 times in sterile phosphate buffer saline (PBS) with antibiotics (Ab) (1% penicillin, amphotericin-B and gentamicin (Biowest)) for 30 min at room temperature.

All assays were performed with bone marrow stromal cells (BMSCs) from Wistar rats. Briefly, BMSCs were expanded in Petri dishes with Dulbecco’s Modified Eagle Medium (DMEM) supplemented with bovine fetal serum (BFS) 10% and Ab and maintained in culture at 37 °C and 5% CO₂.

5 × 10⁴/ml BMSCs were seeded in the scaffolds in 24 well plates in Hank’s Balanced Salt Solution for 4 h at 37 °C and 5% CO₂. As cytotoxicity positive control, cells were exposed to UV light in PBS, while PBS without cells and supernatant from cells without UV light exposure were used as negative control and basal cell death control respectively. After 4 h incubation, 100 µl of supernatant from each sample were taken and placed in a 96 well plate and 100 µl of the cytotoxicity detection kit LDH solution added and incubated for 20 min at room temperature and darkness. Finally, the 96 well plates were analyzed in a spectrophotometer at wavelength 490 nm. Each experiment was performed in triplicate. To determine the percentage
of cytotoxicity the resulting values of absorbance were substituted in the following equation:

\[
\text{Cytotoxicity (\%)} = \left( \frac{\text{experimental average absorbance} - \text{absorbance of live cells number}}{\text{absorbance dead cells number} - \text{absorbance live cells number}} \right) \times 100
\]

For cultures \textit{in vitro}, 5 × 10^4/ml BMSCs in DMEM supplemented medium were gently injected in the scaffolds with a 0.3 mm × 13 mm needle, medium was replaced every other day during seven days. Scaffolds with cells were fixed with 10% formalin solution for 2h and included in paraffin in order to obtain 5µm slices that were stained with H-E for histological analysis. Lastly, photomicrographs were taken with a Nikon Eclipse 80i microscope.

\section*{Statistic analysis}

Repeated measures analysis of Kruskal-Wallis was performed. A P value <0.05 was considered statistically significant. All analyzes were performed in the GraphPad Prism 6 software.

\section*{Results and Discussion}

\subsection*{Scaffold production}

Combining EDC at 6, 30, 50 or 60 mM with a mixture of 4% Ge and 1% HA, porous sponge-like scaffolds were obtained (Figure 1). The EDC crosslinking mechanism \cite{26,27} depends of the reaction with carboxyl groups of Ge and/or HA, which produce activated O-urea, that may react with the amino groups of Ge, resulting in the amide bond formation and the release of soluble urea (Figure 2).

In addition, EDC may be easily deactivated in aqueous solution and 1% HA, porous sponge-like scaffolds were obtained (Figure 1). The EDC crosslinking mechanism \cite{26,27} depends of the reaction with carboxyl groups of Ge and/or HA, which produce activated O-urea, that may react with the amino groups of Ge, resulting in the amide bond formation and the release of soluble urea (Figure 2).

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were made. The great forming gelatin films capacity may contribute to the orientation of the pore channels in the superior part of scaffolds. On the other hand, the inferior part of the scaffolds had different forms due to the fact that vacuum was no homogeneous in the superior and inferior part of the scaffold.

It is important to notice that the number and pore size, are essential for cell growing, proliferation and adhesion, inducing the development of tissues and tissue regeneration [31]. As a result, it has been considered that a pore size of 40 to 200 µm is appropriate to create an adequate microenvironment for cell development.

Absorption capacity

In order to have a suitable scaffold for cell proliferation, it should have certain hydrophilic capacity, which may provide an appropriate contributions of nutrients dissolved in liquids, good oxygenation capacity and fluid body loss prevention during a patient treatment [28], for this reason in this study the absorption water capacity of sponges obtained after different concentrations of EDC was analyzed. The absorption water capacity in weight of every sponge made with different concentrations of EDC was obtained by subtracting the initial weight from the final weight of the scaffolds after the immersion in distilled water for one hour. The results of this experiment showed that the maxim water absorption was in the sponges manufactured without EDC. The water absorption capacity was decreased as the concentration of EDC was diminished (Figure 5). It has been reported previously [32] that when the crosslinkers concentration increased, decreases the absorption water capacity due to the development of networks between polymer chains in the scaffolds after been treated with different crosslinkers. So, if lower concentration of crosslinker is used, less polymer networks will be formed and a greater amount of free chains will be found. Furthermore, the hydrophilic capacity of hyaluronic acid is still preserved even if it is crosslinked with EDC.

Energy-dispersive X-ray spectroscopy (EDS)

In order to know the chemical composition of Ge/HA scaffolds crosslinked with different concentrations of EDC, elements in each sample were analyzed by EDS. Figure 6 shows EDS analysis, in logarithmic scale, of the Ge/HA scaffolds. All scaffolds showed the presence of various elements such as carbon, oxygen, sodium, chloride, sulfur and calcium. Figure 6G indicates the concentrations of several elements in scaffolds expressed in weight percent (wt%). The concentration of some elements in the sponges did not change significantly; nonetheless, is important to mention that concentration of sodium and sulfur changed in the scaffolds, not significant but taken into account. Both sodium and sulfur concentration were decreased as EDC concentration used in scaffolds increased. It is known that HA has no sulfur groups and therefore, although it is highly hydrophilic, it is less than other glycosaminoglycans. Nevertheless, when HA is combined with Ge the sulfur groups appeared, indicating that they were derivated from the Ge in a proportion of 0.80 in the sponges crosslinked with 6 mM of EDC; in contrast, sponges crosslinked with 60 mM of EDC showed a sulfur groups proportion of 0.06. The fact that the Ge/
HA sponges crosslinked with 60 mM of EDC presented less sulfur proportion is consistent with their reduced ability to attract water as it has already mentioned above. The ability of glycosaminoglycans to absorb water depends of the presence of negative sulfur molecules, which causes the attraction of sodium ions and water to connective tissue rich in proteoglycans. The fact that the Ge/HA sponges may have shown to possess sulfur groups suggests that Ge/HA scaffolds may be suitable for cell growth and proliferation in order to form tissues.

**Cytotoxicity**

With the objective of analyze if the crosslinking process of Ge/HA is suitable for cell growth, a cytotoxicity test was conducted by quantifying the releasing of LDH enzyme colorimetrically. The LDH detection method in culture medium as indicator of cytotoxicity is a reliable and easy method, frequently used in tissue engineering [33].

The culture supernatant in all cases was obtained 4 h after the cells were seeded on the scaffolds crosslinked with different concentrations of EDC. Cytotoxicity results demonstrated that LDH concentration (color diminished) decreased as fewer amounts of dead cells were found in each experiment and control groups. Thus, it was observed that the maximum absorbance, 0.68, was in positive control groups (dead cells were seeded on scaffolds), the lower absorbance, 0.2, was the sample with live cells seeded on scaffolds, and the culture medium without cells did not show any absorbance signal. The cellular percentages of cytotoxicity were obtained following the equation described above in the methodology section. The results of the percentage cytotoxicity obtained were as follow: 6 mM of EDC= 20%, 30 mM of EDC= 44%, 50 mM of EDC= 40% and 60 mM of EDC= 94% of cellular percentages of cytotoxicity (Figure 7).

In all cases, any of the scaffolds crosslinked with different concentration of EDC induced cytotoxicity (all groups showed significant difference compared with control group of dead cells), with the exception of the scaffold made with 60 mM of EDC, that did not show significance with respect the control of dead cells. The fact that the Ge/HA scaffolds crosslinked with 60 mM of EDC induced cytotoxicity may be due to a inefficient waste removing method, as others have noted [23], or perhaps it may be necessary a longer lyophilization process in order to remove the crosslinker.

**In vitro cultures**

The scaffolds crosslinked with different concentrations of EDC were seeded with 5 x 10^4 BMSC for 7 days, histological sections were obtained and stained with HE. On scaffolds crosslinked with 6 mM, 30 mM and 50 mM of EDC, cell clusters with eosinophilic cytoplasm, granular chromatin and cytoplasm projections were observed (Figures 8A–8C). Furthermore, it has been noticed on the scaffolds crosslinked with 60 mM of EDC just a few cells with notorious cell damage and small and piknotic nuclei (Figure 8D), signs of cell death. This finding is consistent with results of cytotoxicity, where it was observed that the scaffolds crosslinked with 60 mM of EDC showed increased cytotoxicity (Figure 8E).

Besides the concentration of EDC, cell death may be induced by the pore size of scaffolds and low water and oxygen diffusion into the scaffolds, as a result of the low water absorption capacity of scaffolds crosslinked with 60 mM of EDC, as we have already mentioned above.

**Conclusion**

This study has shown that the crosslinking of Ge and HA with different concentrations of EDC is useful for constructing scaffolds that may be used for diverse applications in tissue engineering. The scaffolds manufactured, depending of their properties of hydrophilicity, size and pore number, may be used with or without cells in order to facilitate the repair of distinctive tissues such as skin, cartilage and bone. In particular, the scaffolds crosslinked with 6, 30 and 50 mM concentrations of EDC may be useful in tissue engineering due to their low cytotoxicity.

**Acknowledgement**

This research was partially supported by project PAPIIT-DGAPA, UNAM, IT201313. The first author is a student of Ph.D. Program "Posgrado en CienciaMédicas, Odontológicas y de la Salud" of the Universidad Nacional Autónoma de México and acknowledges the scholarship and financial support provided by CONACYT, UNAM. The authors thank to Erick Francisco Sánchez Ortega, Dulce María López, Raquel Guerrero Alquicira, Sara Judith Alvarez and Armando Zapeda Rodríguez for expert technical assistance, Christian Cárdenas Ortega, Dulce María López, Raquel Guerrero Alquicira, Sara Judith Alvarez and Armando Zapeda Rodríguez for expert technical assistance, Christian Cárdenas Monroy for language technical advice and Nancy Carolina Piñón Zárate for providing Spanish-English translation and helpful technical advice.

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