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研究課題名（和文）Development of a novel cellulose scaffold to potentiate the transplanted cells survival for bone regeneration

研究課題名（英文）Development of a novel cellulose scaffold to potentiate the transplanted cells survival for bone regeneration

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研究成果の概要（和文）：本研究では新規骨補填材の開発を目指し，メチルセルロースを基材とした細胞移植担体の製造法の至適化を行った．メチルセルロースに異なる架橋密度、多孔性を付与した細胞移植担体の製造を試みたところ，塩化ナトリウムの含有率が担体の均質性と多孔性に大きな影響を及ぼしたが，架橋剤であるカルボニルジイミダゾールの濃度による著名な変化は認められなかった．凍結乾燥の結果，担体の厚みは大幅に減少し，十分な機械的強度を有する構造を付与することは困難であった．メチルセルロースによる細胞移植担体の製造には条件のさらなる至適化が必要である．

研究成果の学術的意義や社会的意義
Increase in the life expectancy and diseases that causes bone loss, has shown an urgent need to improve and optimize bone graft treatment. Development of novel regenerative biomaterials such as Methylcellulose scaffolds, aims to improve our knowledge on the intrinsic mechanism of tissue repair.

研究成果の概要（英文）：In this investigation, we used Methylcellulose (MC) to develop and optimize a novel scaffold material for bone cell transplantation. A series of MC scaffolds with different porosity, cross-link density, and size were fabricated. Results showed that Sodium Chloride (NaCl) has a great effect on the homogeneity and porosity of the scaffold in a dose-dependent manner. Crosslinking using carbonyldiimidazole (CDI) at different concentrations showed no significant changes in the scaffold’s characteristics. As a result of the lyophilization procedure, the thickness of the scaffold was significantly reduced; consequently, affecting the scaffold's structure and compromising the mechanical strength needed for tissue transplantation. Although the production of MC scaffolds was achieved, homogeneity between samples was rather difficult to obtain. Thus, further optimization is required for the production of viable cell transplantation scaffolds using methylcellulose.

研究分野：生体歯科補綴

キーワード：Methylcellulose　Scaffolds　Mesenchymal Stem cells　Bone Regeneration

１．研究開始当初の背景/Background at the beginning of research

Tissue and bone regeneration are methods and procedures under intense study because of its potential applications in clinical treatments. In dentistry, it has been used to facilitate bone augmentation and wound healing. Moreover, an increase in the life expectancy as well as the high incidence of diseases that causes bone loss, such as infections, tumors and trauma, has shown an urgent need to improve and optimize bone graft treatment, particularly in elderly people where regenerative capacity is significantly decreased compared to young people.[1]

Currently, the potential use of cell-based therapy to facilitate bone regeneration in the treatment of dental implant wound healing and alveolar bone defects is of much interest and scrutiny. [2,3] Bone regeneration through the use of Mesenchymal Stem Cells (MSCs) has been studied and used as a viable therapy. [4–6]

In a previous report using different sources of MSCs we demonstrated that culture conditions have great influence in the differentiation and mineralization of the MSCs. [7] Furthermore, cell containing transplants accelerated bone formation, however, survival of transplanted cell did not fully explain bone formation and mineralization.[5] Thus, the use and development of regenerative biomaterials such as scaffold as a delivering system, aims to improve the repair of damaged tissues by influencing or controlling the function and fate of the cells. Nevertheless, the study of other factors that can further contribute to the improvement of bone formation are needed. Collagen scaffold for the delivery of the cells into the defect has proven to further regulate bone formation; however, drawbacks like its weak mechanical properties, source origin (usually a bovine xenograft material) and process, prompt the need and search for suitable alternatives; with cellulose, as a potential alternative.

Potential of cellulose scaffolds in cell-based bone augmentation.

Scaffolds are materials engineered to promote cellular interactions and facilitate the formation of new functional tissues. Scaffolds aim to mimic the extracellular matrix of the native tissue allowing cells to influence their own microenvironments. A variety of synthetic and naturally derived scaffolds have been developed to provide a similar structural support for the cells to migrate, regulate, proliferate and differentiate, leading to the formation of the target tissues.

The most abundant fibers found in nature are collagen and cellulose Fig.1. Collagen is mainly present in animal tissues while cellulose is one of the major constituents in plant cell walls.[8]

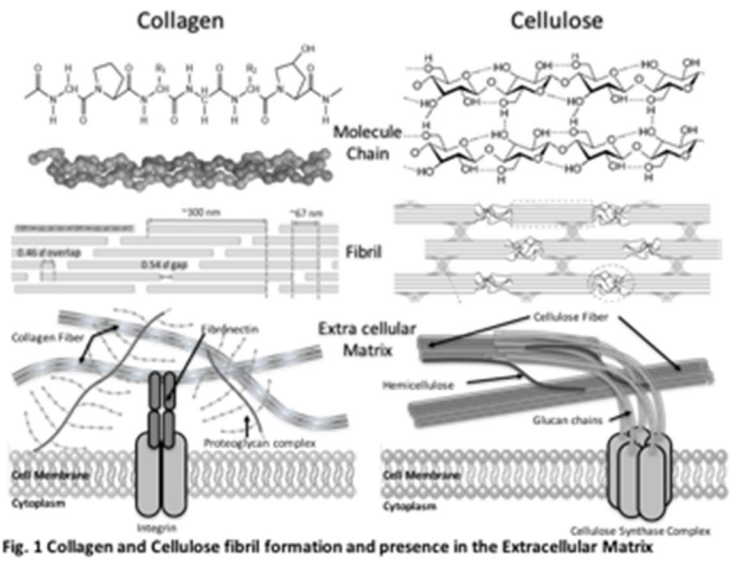

Fig. 1 Collagen and Cellulose fibril formation and presence in the Extracellular Matrix

Collagen is the major constituent of the bone extracellular matrix and it has long been used in tissue engineering. However, the disadvantages of collagen as a biomaterial include its low level of biomechanical stiffness and rapid rate of biodegradation. Although it can be chemically modified to

make up for these disadvantages, it implies additional manufacturing steps that might compromise structural integrity and increase costs. Moreover, the possibility of zoonosis and adverse immune-reactions have been documented.

Cellulose, in the other hand, is a linear homopolymer of glucose with many advantages. It can be obtained from many sources, including bacteria and algae, therefore is easily accessible, cost effective and suitable for industrial quantities[9]. The biocompatibility of cellulose and its derivatives has been well established; Methylcellulose (MC), for example, is often used as a good food additive for food thickening approved by the FDA. Therefore, cellulose based scaffold would be a great alternative to current collagen based ones.[10,11]

２．研究の目的 / Purpose of research

Current standard cell-based therapy for bone augmentation involves autologous bone transplantation; where tissue is harvested from the patient, expanded in vitro and then transplanted into the damaged site.[12] In order to transplant the cells into the site, generally a collagen base scaffold is used. However, due to its low level of biomechanical stiffness and rapid rate of biodegradation as well as the possibility of zoonosis and immune-reactions[13], a better substitute is necessary. In this study we used Methylcellulose as an alternative to collagen-based scaffolds. Due to the presence of abundant hydroxyl groups, Methylcellulose can be used to prepare scaffolds with varying structures and properties to act as a platform for regenerative treatment. Moreover, mixtures of methylcellulose with substances with gelling properties, like agarose, have shown to change the properties of methylcellulose and provide to the mixed hydrogel with regenerative abilities.[14] Thus, the purpose of this study was to research and determine a suitable blend of MC and hydrogel polimers that could allow the fabrication of a suitable MC scaffold for tissue regeneration.

３．研究の方法 / Research method

Preparation of the scaffolds.

For the preparation of the MC-scaffold, procedures previously described by Shen el al. [15] was used as a guideline.

Methylcellulose 500mg (M7140 Sigma-Aldrich) was mixed with 50 mg of Gelatin (G9391 Type B powder, Sigma-Aldrich) and 50 mg of Agarose (A9045, Sigma-Aldrich) in 25 mL dimethyl sulfoxide (DMSO) (D4540, Sigma-Aldrich) to a homogeneous solution using an orbital shaker with a circular shaking motion at a slow speed. After mixing, ground-NaCl in concentration of 5, 2.5 and 0 grams were added into the solution, and vigorous stirred for 4 h.

Then, 500 mg of cross-linking agent; Carbonyldiimidazole (CDI) (sigma-Aldrich)was added into the homogenized mixed solution and then vigorously agitated for 15 min.

Cross-linking agent (carbonyldiimidazole, CDI) was added into the homogenized mixed solution at different concentration, 250 mg for low degree of cross-linking (LCL), 500 mg for medium degree of cross-linking (MCL) and 750 mg for high degree of cross-linking (HCL); the solution was then vigorously agitated for 15 min.

The solutions were then poured into 35mm, 6 well, 24 well and 96 well dishes that serve as molds to give a uniform size and shape to the scaffolds and incubated in ice bath for scaffold formation for 15 min. Once gel was set, samples were then washed with distilled water 5 times to remove NaCl, DMSO and unreacted reagents. Washed samples were kept at -80˚C overnight.

Scaffold processing.

There are numerous approaches to fabricate porous scaffolds from natural polymers, the most commonly used techniques involve phase separation, solid freeform fabrication, bioprinting, supercritical fluid technology, porogen leaching, freeze drying, electrospinning, centrifugal casting, scaffold templating techniques, and micro-patterning techniques. Of these, freeze-drying, electrospinning, and also gelation are mostly used. For this experiment, lyophilization using a freeze dry machine (VD-500F) to obtain the MC-Scaffolds was chosen as the most suitable process for our mixed solution. (Fig. 2).

4．研究成果/ Research result

In this investigation, a series of porous structured MC-based scaffolds with different cross-linking density, porosity and size were fabricated.

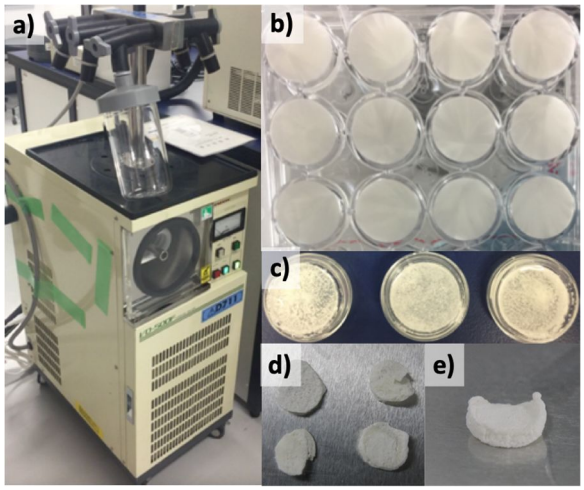

Fig. 2 a) Lyophilization machine. b) Frozen MC-gel scaffolds (-80℃) before freeze-dry. c) 35mm well dish lyophilizated MC-scaffold. d) De-molded 96 well dish sized MC-scaffolds. e) MC-scaffold thickness.

Mixed Solution.

MC in water requires temperature to completely dissolve; with DMSO however, only gentle oscillation is needed. Gel and Agar, in the other hand, require a stronger oscillation and longer time to completely dissolve. However, agitation seems to affects the strength and apparent temperature of gelation; moreover, continued rapid agitation during gelation will break down the gel structure.

Porosity

Although DMSO is considered one of the strongest organic solvents, during preparation, we observed that NaCl crystals did not dissolved homogenously at the concentration indicated in the original protocol, moreover, it tended to precipitate if left unstirred. Distribution of the salt crystals prove to be difficult within the solution even after a prolonged and constant stirring. Nevertheless, better homogeneous concentration was obtained by mixing the solution with NaCl in the molds.

NaCl prove to have a great influence in the scaffold's consistency and porosity. Sample with no salt (0 grams control) showed no porosity and remained in a gel like state, increased concentration (2.5 grams and 5 grams) showed an increased porosity in a dose dependent manner. However, the high concentration of salt, also influenced the freezing point of the samples, therefore NaCl, DMSO and unreacted chemicals were removed with distilled water before freezing at -80C.

Cross linking.

Hydrogels are basically networks of cross-linked polymers, thus in this experiment Carbodiimides (CDI) was used to mediate the formation of linkages. CDI is probably the most popular type of crosslinker in use, being efficient in forming conjugates between two protein molecules, between a peptide and a protein, between an oligonucleotide and a protein, between a biomolecule and a surface or particle, or any combination of these with small molecules.[16] CDI was added at concentrations of

250, 500 and 750 mg (low, medium and high respectively) to adjust gel crosslink. Result didn't show any apparent difference between groups at these concentrations.

Lyophilization.

During lyophilization, several gels samples developed crevasses, these were more likely the result of the vacuum during the process that tended to pull the samples out of the molds compromising the integrity of the scaffold. In addition, demolding also produced fissures in some of the samples.

After lyophilization, thickness was considerably reduced. Samples were prepared with a thickness of about 5 to 7mm; after procedure, these were reduced to about 2-3mm. Consistent thickness throughout all the samples was difficult to achieve.

Most importantly, porosity of the scaffolds was not homogenous nor consistent throughout the preparations.

Conclusion.

Although MC scaffold were achieved, thickness, pore size and salinity were difficult to standardize. An important aspect to consider in biomaterial engineering is sample consistency. Biomaterial physical characteristics greatly influence its properties and interactions with the surrounding environment. Discrepancies between samples greatly affect tissue interactions such as cell proliferation and differentiation; and therefore, also impact the result outcomes. The MC scaffold obtained in this experiment require more testing in order to standardize the samples. Despite these shortcomings, MC as scaffold have a great potential for tissue regeneration and more tests and analysis are required.

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５．主な発表論文等

〔雑誌論文〕　計3件（うち査読付論文　2件／うち国際共著　1件／うちオープンアクセス　2件）

１．著者名 Rocabado Juan Marcelo Rosales、Kaku Masaru、Nozaki Kosuke、Ida Takako、Kitami Megumi、Aoyagi Yujin、Uoshima Katsumi	４．巻 14
２．論文標題 Response to Letter to the Editor: Concerns on modeling postmenopausal osteoporosis in young female rats	５．発行年 2019年
３．雑誌名 Journal of Orthopaedic Surgery and Research	６．最初と最後の頁 451
掲載論文のDOI（デジタルオブジェクト識別子） 10.1186/s13018-019-1485-2	査読の有無 無
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

１．著者名 Rosales Rocabado Juan Marcelo、Kaku Masaru、Nozaki Kosuke、Ida Takako、Kitami Megumi、Aoyagi Yujin、Uoshima Katsumi	４．巻 13
２．論文標題 A multi-factorial analysis of bone morphology and fracture strength of rat femur in response to ovariectomy	５．発行年 2018年
３．雑誌名 Journal of Orthopaedic Surgery and Research	６．最初と最後の頁 318-328
掲載論文のDOI（デジタルオブジェクト識別子） 10.1186/s13018-018-1018-4	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

１．著者名 Ida Takako、Kaku Masaru、Kitami Megumi、Terajima Masahiko、Rosales Rocabado Juan Marcelo、Akiba Yosuke、Nagasawa Masako、Yamauchi Mitsuo、Uoshima Katsumi	４．巻 13
２．論文標題 Extracellular matrix with defective collagen cross-linking affects the differentiation of bone cells	５．発行年 2018年
３．雑誌名 PLOS ONE	６．最初と最後の頁 1-18
掲載論文のDOI（デジタルオブジェクト識別子） 10.1371/journal.pone.0204306	査読の有無 有
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〔学会発表〕　計0件

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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共同研究相手国	相手方研究機関