氏名（本籍地）	Qin Zhang（中国）
学位の種類	博士（工学）
学位記番号	博甲第6674号
学位授与年月日	平成25年7月25日
学位授与の要件	学位規則第4条第1項該当
審査研究科	数理物質科学研究科
学位論文題目	Preparation of Porous Scaffolds with Controlled Pore Structures for Tissue Engineering （組織再生のための空孔構造を制御した多孔質足場材料の作製）

主査	筑波大学教授	博士(工学)	青柳 隆夫
副査	筑波大学准教授	博士(農学)	辻村 清也
副査	筑波大学准教授	博士(工学)	田口 哲志
副査	筑波大学准教授	博士(工学)	陳 国平

論　文　の　要　旨

Appropriate pore structures are required for the scaffolds that are used for tissue engineering and regenerative medicine. Although a number of three-dimensional porous scaffolds have been developed from various types of biodegradable polymers, it has been difficult to ensure homogenous tissue formation in the scaffolds because of uncontrolled pore structure and poor interconnectivity. In general, cells are easily allocated and distributed in the peripheral areas, resulting in partial tissue regeneration in the outermost peripheral layers of the scaffolds. The regeneration of functional tissues requires smooth cell delivery and distribution throughout the scaffolds.

To improve spatial cell distribution and promote homogeneous regeneration, some preparation methods have been developed for controlling various aspects of the pore structures, such as pore size, porosity and interconnectivity of the scaffolds. Among these methods, porogen-leaching method offers many advantages for the easy manipulation and control of pore size and porosity. Although the porogen materials can leave replica pores after leaching, they cannot initiate the formation of surrounding pores. As a result, isolated pores are formed in the scaffolds, a situation which is not desirable for tissue engineering scaffolds. To improve pore interconnectivity, the porogen materials are bonded before mixing them with polymer matrix. However, the bonded porogen materials require organic solvents for leaching of the porogen materials, and the residual solvents are toxic to cells. Penetration of the polymer solution into the bonded porogen material becomes difficult if the polymer solution has a high viscosity. Therefore, development of easy and effective methods for preparation of porous scaffolds with well controlled pore structures is strongly desirable.

In this study, we developed a novel method by using pre-prepared ice particylates as porogen materials to precisely control the pore structures of collagen-based porous scaffolds. At first, the method was established by mixing the pre-prepared ice particulates with collagen aqueous solution at different mixture

ratios and concentrations and the detailed preparation conditions were investigated. Subsequently, the method was used to prepare a collagen scaffold with a gradiently changed pore size to investigate the effect of pore size on cartilage tissue regeneration. Furthermore, individual collagen porous scaffolds with different pore sizes were prepared to compare the pore size effect on proliferation and cartilaginous gene expression of chondrocytes. Finally, the method was used to prepared hybrid scaffolds of collagen and wollastonite nanowires with precisely controlled pore structures for bone tissue engineering.

(1) Preparation of collagen scaffolds with precisely controlled pore structures by using ice particulates as a porogen material.

Precisely controlled pore structures are required for the scaffolds to control cell distribution and functions for functional tissue regeneration. In this part, a method using pre-prepared ice particulates as a porogen material was developed to precisely control the pore structures of collagen porous scaffolds. The detailed preparation conditions were investigated. Ice particulates were prepared by spaying Milli Q water into liquid nitrogen using a sprayer. The ice particulates were sieved by sieves with 335 and 425 μm mesh pores to obtain ice particulates having a diameter from 335 to 425 μm. Two groups of collagen porous scaffolds were prepared. Group A: the collagen scaffolds were prepared using a 2 % (w/v) collagen aqueous solution with a ratio of ice particulates/collagen solution of 25 %, 50 % and 75 % (w/v) at a freezing temperature of -80 °C. Group B: the collagen porous scaffolds were prepared using 1 %, 2 % and 3 % (w/v) collagen aqueous solution with a ratio of ice particulates/collagen solution of 50 % (w/v) at a freezing temperature of -80 °C. The preparation scheme is as follows. First, the collagen solution was prepared by dissolving freeze-dried porcine type I collagen in a solution (20:80 (v/v)) of ethanol and 0.1 M acetic acid (pH 3.0) at 4 °C. The solution of acetic acid and ethanol was used to dissolve collagen to decrease the freezing temperature of the collagen solution below -4 °C. Before mixing, the ice particulates and collagen solution were kept in a -4 °C, low-temperature chamber for 6 h for temperature balance. Subsequently, the ice particulates were added to the collagen solution and mixed thoroughly with a steel spoon to ensure that the ice particulates and collagen solution were homogeneously mixed. The mixture was poured onto a perfluoroalkoxy (PFA)-film wrapped copper plate with a 10 mm thick silicone frame, and then the mixture surface was flattened with a steel spatula and covered with a glass plate wrapped with polyvinylidene chloride film. The manipulation was conducted in the -4 °C, low-temperature chamber. After mixing, the whole set was moved to a deep freezer (-80 °C) and kept there for 6 h of freezing. The frozen mixture was freeze-dried for 3 days in a freeze-dryer under a vacuum of 20 Pa to form collagen porous scaffolds. Finally, the freeze-dried collagen porous scaffolds were cross-linked for 6 h with glutaraldehyde vapor, which was saturated with a 25% aqueous glutaraldehyde solution at 37 °C in a closed box. After cross-linking, the scaffolds were immersed in a 0.1 M glycine aqueous solution to block any unreacted aldehyde groups. The scaffolds were washed 6 times with pure water. The washed scaffolds were frozen in a deep freezer (-80 °C) for 6 h and freeze-dried again as described above to obtain the dried collagen scaffolds. The collagen scaffolds prepared by ice particulates were defined as ice-collagen scaffolds. The control collagen scaffold was prepared by the same procedure with a 2 % (w/v) concentration of collagen solution without

the use of ice particulates. The pore structures of scaffolds were observed by SEM and mechanical properties were investigated by a static mechanical test. Bovine articular chondrocytes were cultured in the ice-collagen scaffolds and control scaffold to compare their effects on regeneration of cartilage tissue. The results showed that ice particulates with a diameter range from 355 μm to 425 μm were obtained and the ice-collagen porous scaffolds showed different appearance to that of control collagen scaffolds. The ice-collagen scaffolds had interconnected large spherical pores surrounded by small pores. The large pores were spherical and were the same size as the ice particulates. The ice-collagen scaffold prepared with 2% collagen and 50% ice particulates showed the most homogeneous cell distribution due to its homogeneous pore structure. Young's modulus of the ice-collagen scaffold increased 15.7-fold compared with that of control collagen scaffold prepared. The ice-collagen scaffold with precisely controlled pore structure and high mechanical property supported chondrogenesis more strongly than did the control scaffold. These results suggest that the method will be useful for the preparation of collagen-based porous scaffolds for tissue engineering and regenerative medicine.

(2) Preparation of collagen porous scaffolds with a gradient pore size structure.

Some cell functions as well as new tissue regeneration deeply rely on the size of the pores. The use of a gradient pore structure may provide a useful and practical tool to compare the effect of different pore sizes under the same culture conditions. Therefore, collagen porous scaffolds with a pore size gradient were prepared by using pre-prepared ice particulates as a porogen material. At first, an aqueous collagen solution (2% (w/v)) in a mixture of ethanol and acetic acid (20:80 v/v, pH 3.0) was prepared. The ice particulates were prepared by spraying Milli Q water into liquid nitrogen using a sprayer. The ice particulates were sieved by sieves with mesh pores of 150, 250, 355, 425 and 500 μm to obtain ice particulates having diameters of 150-250, 250-355, 355-425, and 425-500 μm. Then, the aqueous collagen solution was mixed with the sieved ice particulates in a 50:50 (v/w) ratio at a -4 °C low-temperature chamber. Each of the four mixtures of collagen solution and ice particulates of different diameters was poured into a silicone frame that was then placed on a PFA film-wrapped copper plate, and the mixture surface was flattened with a steel spatula. The four mixtures in their frames were stacked together with ice particulate sizes increasing from bottom to top. Finally, the entire set was freeze-dried, cross-linked and blocked as described in Part One. The gradient pore scaffolds were used for culture of bovine articular chondrocytes, directly examining the effect of pore size on cartilage regeneration. The results showed the gradient collagen scaffolds had well-interconnected pore structures with compactly packed spherical pores. Chondrocytes adhered and showed a homogenous distribution throughout the scaffolds. HE staining showed that the spatial cell and ECM distribution was uniform and that tissue formation was homogeneous in the gradient collagen scaffold after 8 weeks of *in vivo* implantation. The scaffolds prepared with ice particulates in the range of 150-250 μm showed the most compact and abundant GAG production by the chondrocytes and most strongly stained type II collagen. The micropores in the scaffolds prepared with ice particulates in the range of 150-250 μm showed the most beneficial effect on cartilage regeneration. Gradient scaffolds prepared with ice particulates were a useful tool to directly compare the effects of scaffold pore size on tissue

regeneration.

(3) Preparation of collagen porous scaffolds with different pore sizes.

Scaffold pore size was shown in Part Two to be an important factor affecting tissue regeneration efficiency. To further compare the effect of pore size on the proliferation and gene expression of chondrocytes, four individual ice-collagen porous scaffolds with different pore sizes were prepared by the method described in Part One. Ice particulates with diameters of 150-250, 250-355, 355-425 and 425-500 μm were separately mixed with collagen aqueous solution in a 50:50 (v/w) ratio. Each mixture was frozen at -80 °C, freeze-dried, cross-linked and blocked as described above to obtain the individual collagen scaffolds. The sizes of the large pores in the ice-collagen scaffolds were in a good agreement with the sizes of the prepared ice particulates. Bovine articular chondrocytes cultured in the individual ice-collagen scaffolds showed homogeneous distribution throughout all the four scaffolds. The cell/scaffold constructs were subcutaneously implanted into nude mice for 8 weeks. The DNA and sGAG contents of the cell/scaffold constructs after 6 hours and 1 week of *in vitro* culture and after 8 weeks of *in vivo* implantation were measured. The DNA amount increased almost 4-fold after 1 week of *in vitro* culture and almost 6-fold after 8 weeks of *in vivo* implantation. The amount of sGAG increased significantly after 8 weeks of *in vivo* implantation. The sGAG/DNA ratio increased significantly from 1 week of *in vitro* culture to 8 weeks of *in vivo* implantation. The sGAG/DNA ratio in the collagen scaffold prepared with 150-250 μm ice particulates was significantly higher than that in the collagen scaffolds prepared with the 250-355, 355-425 and 425-500 μm ice particulates. The expressions of genes encoding type II collagen and aggrecan were analyzed by real-time RT PCR. Compared to P1 chondrocytes, the chondrocytes in the collagen scaffolds showed higher expression levels of the *Col2a1* and *Acan* genes after 8 weeks of implantation. The chondrocytes cultured in the collagen scaffold with the smallest pores expressed the highest levels of *Col2a1* and *Acan* among all the collagen scaffolds. The Young's moduli of the implants after 8 weeks of implantation were measured. The collagen scaffold implant prepared with 150-250 μm ice particulates had the highest Young's modulus. These results indicated that the collagen porous scaffolds prepared with ice particulates in the range of 150-250 μm showed the most promotive effect on the gene expression and the production of cartilaginous matrix proteins as well as on cartilage regeneration.

(4) Preparation of hybrid porous scaffolds of collagen and wollastonite nanowires.

Hybrid scaffolds of collagen and wollastonite nanowires with well controlled pore structures were prepared by using ice particulates having a diameter from 335 to 425 μm. The collagen/wollastonite nanowires solution was obtained by homogeneously mixing collagen aqueous solution and wollastonite nanowires solution. The final collagen concentration in collagen/wollastonite nanowires solution was 2% (w/v) and the weight ratio of wollastonite to collagen was 10:90 (w/w). The temperature-balanced ice particulates were added into the collagen/wollastonite nanowires solution in a 50:50 (v/w) ratio at -4 °C. The mixture was frozen at -80 °C, freeze-dried, cross-linked with glutaraldehyde vapor and blocked with glycine solution. The control collagen scaffold was prepared by the same procedure but without wollastonite nanowires. The collagen/wollastonite nanowires hybrid scaffold are defined as Col/nCS hybrid

scaffold. The scaffolds were used for three-dimensional culture of human bone marrow-derived mesenchymal stem cells (hMSCs). Cell proliferation, osteogenic differentiation and angiogenesis factor gene expression were investigated. The results showed that the wollastonite nanowires had a wire-like structure with a diameter of 20~30 nm and a length of 250~500 nm. The hybrid scaffolds had interconnected large spherical pores with wollastonite nanowires embedded in the pore walls. The hybrid scaffolds facilitated cell seeding and cell distribution. Compared to collagen scaffold, the hybrid scaffolds showed higher mechanical property, higher cell proliferation and osteogenic differentiation and expressed higher level of genes encoding angiogenesis-related genes. Hybridization of collagen with wollastonite nanowires was proved to be a good strategy for the preparation of bone tissue engineering scaffolds.

In conclusion, a method using pre-preapred ice particulates as a porogen material was developed for preparation of collagen-based scaffolds with precisely controlled pore structures such as pore size and interconnectivity. The method was used to prepare collagen porous scaffolds with a pore size gradient and different pore sizes to compare the effect of pore size on cell functions and cartilage tissue regeneration. The effect of pore size on cartilage tissue formation was directly compared by culturing bovine articular chondrocytes in the gradient collagen scaffolds. The effect of pore size on production and expression of cartilaginous extracellular matrices was compared by using individual scaffolds having different pore sizes. The collagen porous scaffolds prepared with ice particulates in the range of 150-250 μm showed the most promotive effect on the gene expression and the production of cartilaginous matrix proteins as well as on cartilage regeneration. The method was also used to prepare hybrid scaffolds of collagen/wollastonite nanowires for bone tissue engineering. The hybrid scaffolds could facilitate osteogenic differentiation and induce angiogenesis when hMSCs were cultured in the scaffolds. The ice particulates method was demonstrated to be a useful method to prepare collagen-based porous scaffolds and its hybrid scaffolds for tissue engineering.

審　　査　　の　　要　　旨

〔批評〕

本論文では、生体吸収性天然高分子であるコラーゲンの多孔質足場材料における空孔構造を制御するために、予め作製した氷微粒子をポローゲンとする新規な作製方法を開発した。空孔構造を制御したコラーゲン多孔質足場材料を作製するために、まずコラーゲン水溶液の濃度や氷微粒子とコラーゲン水溶液の混合比などの条件を検討した。本方法で作製したコラーゲン多孔質材料は連通した球状の空孔を持ち、高い力学強度を示すことを明らかにした。さらに、濃度２(wt/v)％のコラーゲン水溶液及び氷微粒子/水溶液の混合比50/50が最適な作製条件であることが分かった。次に、空孔サイズがそれぞれ150-250、250-355、355-425、425-500 μｍのように傾斜的に変化するグラジエント多孔質構造を有するコラーゲン多孔質足場材料を作製した。本足場材料を用いてウシ関節軟骨細胞を培養し、空孔サイズによる軟骨組織再生への影響を調べた。これを個別に作製した単一空孔サイズのコラーゲン多孔質足場材料による軟骨組織再生への影響と比べた。その

結果、150-250μmの氷微粒子で作製したコラーゲン多孔質材料は軟骨組織再生に最も有効であることが分かった。さらに、この方法でコラーゲンとウォラストナイトナノワイヤーの複合多孔質足場材料を作製した。本複合材料はヒト骨髄由来の間葉系幹細胞の骨分化を促進し、血管形成を誘導する効果を示し、骨組織再生の材料として有効であることが分かった。本論文で開発した方法および作製した材料は新規性をもち、軟骨や骨などの組織再生のための有用な足場材料であり、生体組織工学の発展に重要な知見を与えるものと考える。よって、本論文は博士（工学）の学位論文として十分な学術的価値をもつものと認める。

〔最終試験結果〕

平成25年6月14日、数理物質科学研究科学位論文審査委員会において審査委員の全員出席のもと、著者に論文について説明を求め、関連事項につき質疑応答を行った。その結果、審査委員全員によって、合格と判定された。

〔結論〕

上記の論文審査ならびに最終試験の結果に基づき、著者は博士(工学)の学位を受けるに十分な資格を有するものと認める。