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Preparation and Evaluation of Collagen I/ Gellan Gum/β-TCP Microspheres as Bone Graft Substitute Materials

Kai-Chi Ku, Ming-Wei Lee, Shyh Ming Kuo, Chun-Hsu Yao, Shwu-Jen Chang

Abstract—**Collagen I is the main component of protein in bone and exhibits many excellent applications in biomedical fields. Gellan gum possesses good biocompatible, biodegradable and good mechanical property, and shows great potentials as tissue engineering scaffold or cell culture substrate. Therefore, the aim of this study was to use collagen I, gellan gum and β-TCP to prepare collagen I/gellan gum/β-TCP microspheres by emulsion method as bone graft substitute materials. The preliminary results showed that collagen I/gellan gum/β-TCP microspheres had particle size distribution between 500-1000 μm in diameter and exhibited better mechanical strength. These microspheres also showed good biocompatibility in cell activity test.**

I. Introduction

The ideal bone generating materials should be biocompatible, osteoconductive and osteoinductive. The materials should also be degradable in concert with new bone growth lest it interferes with the new bone formation. In the past few years, efforts to investigate applications of the composite made of polymer and ceramic materials on bone tissue engineering have been increasing [1-6]. A variety of approaches to the development of biodegradable and bioactive composites for tissue engineering applications are being studied, including combinations of collagen and bioactive glasses in making of different scaffolds [7-11].

Among many commercially available ceramics, hydroxyapatite and β-TCP are frequently used as bone repairing and replacing materials because of their biocompatibility, bioactivity, and non-toxicity. Having the chemical composition close to the mineral composition of natural bone, calcium phosphate ceramics have been extensively employed as a bone substitute and revealed to be an invaluable osteo-integrative material [12-15]. In general, the resorption rate of HAP is slower than β-TCP under normal physiological environment. Consequently, this results to a longer-term ceramic persistency of HAP in the system. From clinical practice and experience, β-TCP ceramics possesses a more favorable resorption pattern and osteo-transduction property, i.e., the ceramic material can be gradually absorbed followed by new bone formation and without compromising the intimacy of bone-implant contact.

Collagen I is the primary component of the ECM in bone tissue. Under normal physiological conditions, collagen can undergo a spontaneous self-assembly reaction and then form fibrous structures. Collagen is widely used as a biomaterial in tissue engineering applications [16-18]. Many research efforts have also focused on how to produce an ordered three-dimensional collagen matrix and demonstrated that cells cultured in these collagen matrixes have a state of growth and differentiation behaviors close to that of the *in vivo* environment [19].

Microspheres, on the other hand, could provide larger surface area for cell growth and possess easy estimation of diffusion and mass transfer behaviors. Therefore, microspheres could be more favorably used as cell or tissue carrier, bone grafting and drug delivery encapsulating material in biomedical field.

In the case of collagen I, its poor mechanical properties actually prevented this unique biomedical material from more practical applications, such as for lengthier on-site duration, in form of microspheres of larger size. Therefore, we attempt to improve the mechanical strength of collagen microspheres. Gellan gum, which is also known as polysaccharide S-60, is produced by a non-pathogenic strain of Sphingomonas elodea. Its main chain consists of four repeating carbohydrates, including two D-glucose, one L-rhamnose, and one D-glucuronic acid. Recently, gellan gum has been investigated as a candidate biomaterial for tissue engineering, guided bone regeneration, drug-carrier matrices, gene delivery agents and cell-carrier materials [20-24]. Gellan gum is characterized by its unique gelling behavior that involves temperature-dependent hydrogen bonding and cation-induced electrical incorporation. In addition, gellan gel showed the compatibility with the human body in the long-term fate [25].

In this study, we take advantage of the specific properties of collagen, β-TCP (with osteoinduction potential) and gellan gum (with rapid gelation) as the components for the preparation of a series of microsphere composites. In the present work, novel bioactive and biodegradable microspheres, composed of β-TCP, collagen I and gellan gum, were fabricated by w/o/w emulsion technique. The materials are intended as scaffolds for bone tissue engineering applications. In addition to the assessing of physical properties of composite microspheres, the biological function of the composites was examined by the cell culture test.

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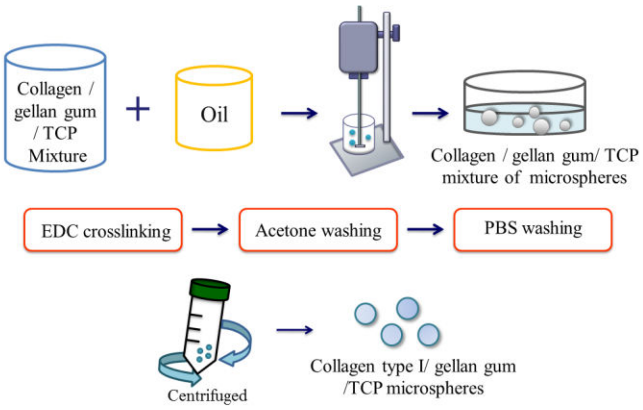
II. Materials and Methods



A. *Preparation of microspheres*

The collagen I/gellan gum/β-TCP microspheres were prepared by the water/oil (w/o) emulsion method [25]. Various weights of gellan gum were dissolved in 100 ml of deionized water and heated at 85-90°C until they became transparent solutions. Collagen I and β-TCP powder were then poured into this solution at 37°C and stirred to allow collagen I and β-TCP powder to be well dispersed. A portion of this mixture was added to the mineral oil. During these processes, this dispersion medium was stirred with a mechanical stirrer at room temperature. About 10 min later, $CaCl_2$ and 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide (EDC) were added into the dispersion medium. Similarly, 50 min later, $CaCl_2$ and EDC were added into the medium and then stirred for 10 min. At the end of period, the collagen I/gellan gum/β-TCP microspheres were washed with acetone to remove mineral oil and collected by centrifugation at 5000 rpm. Then, the microspheres were dried in vacuum and kept in a desiccator for further uses. The prepared collagen I/gellan gum/β-TCP microspheres were observed and examined by microscopy and scanning electron microscopy (SEM).

B. *Cell culture*

The present investigation conformed to the Guide for the Animal Use Protocol of Institutional Animal Care and Use Committee of I-Shou University. The osteoblast cells obtained from neonatal (less than 2 days old) Sprague-Dawley rats were used for the cell culture test because the composites were to be mainly used as bone-related materials.20 Cells between the second and third passages were used in these experiments. Osteoblast cells were first grown in T-75 tissue culture flasks with Dulbecco's modified Eagle medium containing 10% fetal bovine serum (FBS), 50 μg/mL ascorbic acid, and 10 m*M* β-glycerol phosphate. Cells (cell density of 2×10^4 cells/mL) were then transferred to the bacterial grade petri dishes with test samples immersed in the medium.

Cell viability was evaluated using 3-(4,5-dimethyl-thiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) assay and was based on the mitochondrial conversion of the tetrazolium salt. At different time periods, the original medium in each well was replaced with MTT solution (5 mg/ml), and the wells were incubated at 37°C for 4 h to enable the formation of formazan crystals. Then, the solution was removed and DMSO was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes to ensure that all crystals were dissolved, the plates were read on a multi-well scanning ELISA spectrophotometer.

After a particular period, samples were treated with 0.1% Triton X-100, pH 10.5 for one hour. Alkaline phosphatase enzyme was assayed by the method described in Lowry et al.21 by using *p*-nitrophenol phosphate as substrate. Absorbance at 405 nm was read to determine the amount of *p*-nitrophenol produced. The alkaline phosphatase activity of these composites were calculated according to the following equation: ALP activity (m*M*/h)=2×absorbance of composite/absorbance of standard sample, where the standard sample was 1 m*M* *p*-nitrophenol.

III. Results

The preliminary results showed that the microspheres comprised of under 0.5% gellan gum exhibited poor mechanical strength. The microspheres prepared by w/o emulsion method with and without a mixture of β-TCP show mostly sphericity (Fig. 1). These microspheres are about 1000μm in diameter. The incorporation of collagen I and β-TCP did not cause any obviously change in the overall size and shape. (Table 1). The cross-section observation by SEM of microspheres exhibited homogeneous texture for gellan gum-only samples. On the contrary, other microspheres showed extensive heterogeneity of structural morphology inside of the microspheres as shown in Fig. 2. A SEM micrograph revealed that collagen I/gellan gum/β-TCP microspheres exhibited a rougher surface with wrinkles, as shown in Fig. 3. The microspheres remained intact spherical shape after a 3-day mechanical vibration test set at 80 rpm, indicating that these gellan gum-based microspheres exhibited lower agitation-induced fracture frequency compared with the collagen I/β-TCP microcapsules. The preliminary results indicated that the collagen I/gellan gum/β-TCP microspheres with enhanced mechanical strength would be prepared from the very mild cross-linking procedures.

Table 1. Experimental variables used for the preparation of microspheres.

Group	Gellan Gum	TCP (T:G/C, W%)	Collagen (G:T, W%)	Shape	Particle size (μm)	Speed (rpm)
1	1%	X	X	S	800±600	500
2	1.5%	X	X	S	1500±1000	500
3	2%	X	X	S	2000±1000	500
4	1%	2:1	X	S	750±250	750
5	1.5%	2:1	X	S	750±250	1000
6	2%	2:1	X	S	750±250	1400
7	1%	2:1	60:40	S	750±250	750
8	1.5%	2:1	60:40	S	750±250	1000
9	2%	2:1	60:40	S	750±250	1400

S:spherical shape.

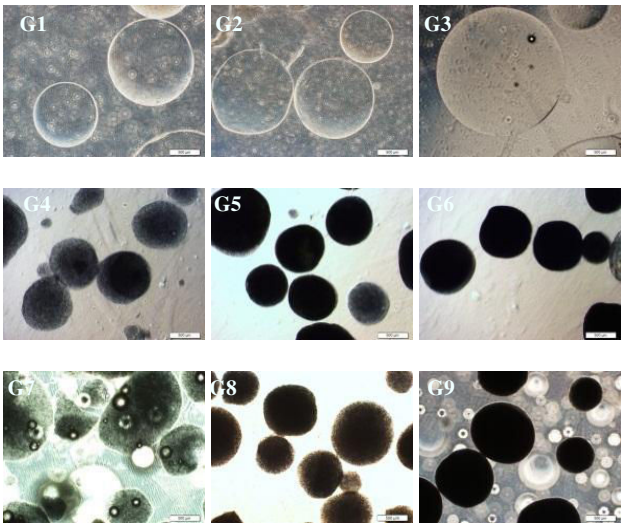


Figure 1. Photomicrographs of the microspheres prepared by emulsion method.

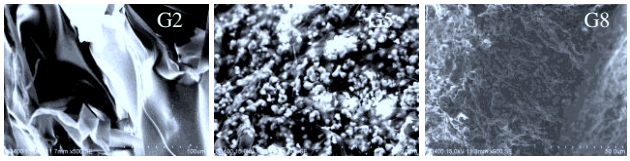


Figure 2. SEM micrographs of the cross-section of the microspheres.

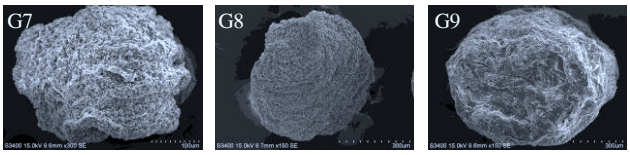


Figure.3. SEM of collagen I/gellan gum/β-TCP microsphere.

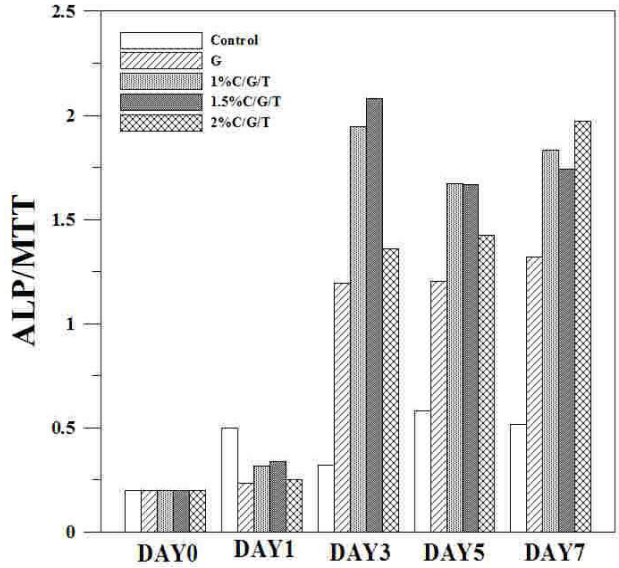


Figure. 4 Alkaline phosphatase activities of osteoblast cells grown on the composite microspheres.

As bone repair or grafting materials, two basic factors were considered in these materials, i.e., these materials should provide appropriate mechanical strength and be able to preserve the function of bone tissues. The phenotype and function of the osteoblasts cells were characterized by the alkaline phosphatase activity. So, in addition to the basic physical properties, the alkaline phosphatase activity of osteoblasts cells was also measured to verify the biocompatibility of these materials. Figure 4 showed the alkaline phosphate activity/MTT in the rat osteoblast cell culture grown on these prepared microspheres. As shown in this figure, the collagen-based microspheres preserved the higher value than the microspheres prepared by only gellan gum after 7 days of culture.

With certain experimental settings, such as the concentrations of the reaction components, the collagen I/gellan gum/β-TCP microspheres with good sphericity were readily prepared. The microspheres were about 500–1000 μm in size. In addition, the mechanical strength and the surface morphology of microspheres could be adjusted. The results indicated that the mechanical strength and the biocompatibility of the microspheres could be enhanced by the gellan gum and collagen, respectively. However, the usage of this new biomaterial, gellan gum, in the application of bone tissue engineering is still needed to evaluate systemically.

REFERENCES

[1] C. Zou, W. Weng, X. Deng, K. Cheng, X. Liu, P. Du, G. Shen, G. Han, "Preparation and characterization of porous β-tricalcium phosphate/collagen composites with an integrated structure", *Biomaterials*, vol. 26, pp. 5276-5284, 2005.

[2] S. J. Chang, S. M. Kuo, C. W. Lan, I. Manousakas, P. H. Tsai, "Evaluation of chitosan/CaSO4/platelet rich plasma microsphere composites as alveolus osteogenesis material", *Biomedical Engineering- Applications, Basic & Communications*, vol. 21, no. 2, pp. 115-122, 2009.

[3] L. C. Lin, S. J. Chang, S. M. Kuo, Gregory Cheng-Chie Niu, Hao Kai Keng, Pei Hau Tsai, "Preparation and Evaluation of β-TCP/Polylactide Microspheres as Osteogenesis Materials", *Journal of Applied Polymer Science*, vol. 108, no. 5, pp. 3210-3217, 2008.

[4] S. M. Kuo, S. J. Chang, L. C. Lin, C. J. Chen, "Evaluating Chitosan/β-Tricalcium Phosphate/Poly(methyl methacrylate) Cement Composites as Bone-Repairing Materials", Journal of Applied Polymer Science, vol. 89, pp. 3897-3904, 2003.09

[5] Susmita Bose, Mangal Roy, Amit Bandyopadhyay, Recent advances in bone tissue engineering scaffolds, Volume 30, Issue 10, Pages 546-554.

[6] Ami R. Amini, Cato T. Laurencin, Syam P. Nukavarapu, Bone Tissue Engineering: Recent Advances and Challenges**,** Critical Reviews™ in Biomedical Engineering, Volume 40, 2012 Issue 5, 363-408.

[7] C. Xu, P. Su, X. Chen, Y. Meng, W. Yu, A. P. Xiang, Y. Wang, "Biocompatibility and osteogenesis of biomimetic Bioglass-Collagen-Phosphatidylserine composite scaffolds for bone tissue engineering", *Biomaterials*, *vol. 32, pp. 1051-1058, 2011.*

[8] S.Yunoki, T. Ikoma, A. Monkawa, K. Ohta, J. Tanaka, "Preparation and characterization of hydroxyapatite/collagen nanocomposite gel", *Journal of Nanoscience and Nanotechnology*, vol. 7(3), pp. 818-21, 2007.

[9] A.A. Sawyer, S.J. Song, E. Susanto, P. Chuan, C.X.F. Lam, M.A. Woodruff, D.W. Hutmacher, S.M. Cool, "The stimulation of healing within a rat calvarial defect by mPCL–TCP/collagen scaffolds loaded with rhBMP-2", *Biomaterials*, vol. 30, pp. 2479-2488, 2009.

[10] Z. Z. Chum, M. A. Woodruff, S. M. Cool, D. W. Hutmacher, "Porcine bone marrow stromal cell differentiation on heparin-adsorbed poly(e-caprolactone)–tricalcium phosphate– collagen scaffolds", *Acta Biomaterialia*, vol. 5, pp. 3305-3315, 2009.

[11] C. Huang, R. Chen, Q. Ke, Y. Morsi, K. Zhang, X. Mo, "Electrospun collagen–chitosan–TPU nanofibrous scaffolds for tissue engineered tubular grafts", *Colloids and Surfaces B: Biointerfaces*, vol. 82, pp. 307-315, 2011.

[12] Q. Fu, N. Zhou, W. H. Huang, D. P. Wang, L. Y. Zhang, H. F.Li, "Effects of nano HAP on biological and structural properties of glass bone cement", *Journal of Biomedical Materials Research Part A*, vol. 74A, pp. 156-163. 2005.

[13] . S. J. Kalita, S. Bose, H. L. Hosick, A. Bandyopadhyay, "CaO–P_2O_5–Na_2O-based sintering additives for hydroxyapatite (HAp) ceramics", *Biomaterials* vol. 25, pp. 2331- 2004, 25, 2331.

[14] N. Matsushita, H. Terai, T. Okada, K. Nozaki, H. Inoue, S. Miyamoto, K. Takaoka,. „A new bone-inducing biodegradable porous β-tricalcium phosphate", *Journal of Biomedical Materials Research Part A*, vol. 70A, pp. 450-458, 2004.

[15] T. Okuda K., Ioku, I. Yonezawa, H. Minagi, G. Kawachi, Y. Gonda, H. Murayama, Y. Shibata, S. Minami, S. Kamihira, H. Kurosawa T., Ikeda, "The effect of the microstructure of β-tricalcium phosphate on the metabolism of subsequently formed bone tissue", *Biomaterials*, vol. 28, pp. 2612-2621, 2007.

[16] J. A. Gilbert, A. E. Simpson, D. E. Rudnick, D. H. Geroski, T. M. Aaberg Jr. and H. F. Edelhauser, "Transscleral permeability and intraocular concentrations of cisplatin from a collagen matrix," *J. Control. Release*, 89: 409-417, 2003.

[17] H. P. Hsu, J. M. Zanella, S. M. Peckham and M. Spector, "Comparing ectopic bone growth induced by rhBMP-2 on an absorbable collagen sponge in rat and rabbit models," *J. Orthop. Res.*, 24: 1660-1669, 2006.

[18] J. Wang, I. L. Lee, W. S. Lim, S. M. Chia, H. Yu, K. W. Leong and H. Q. Mao, "Evaluation of collagen and methylated collagen as gene carriers," *Int. J. Pharm.*, 279: 115-126, 2004.

[19] L. Besseau, B. Coulomb, C. Lebreton-Decoster and M. M. Giraud-Guille, "Production of ordered collagen matrices for three-dimensional cell culture," *Biomaterials*, 23: 27-36, 2002.

[20] S. J. Chang, S. M. Kuo, W. T. Liu, N. C. C. Greory, M. W. Lee, C. S. Wu. "Gellan gum film for effective guided bone regeneration", *J Med Biol Eng,* vol. 30(2), pp. 99-103, 2010.

[21] M. Ahuja, M. Yadav, S. Kumar, "Application of response surface methodology to formulation of ionotropically gelled gum cordia/gellan beads", *Carbohyd Polym,* vol. 80(1), pp. 161-167, 2010.

[22] R. Goyal, S. K. Tripathi, S. Tyagi, K. R. Ram, K. M. Ansari, P. Kumar, "Gellan gum blended PEI nanocomposites as gene delivery agents: Evidences from in vitro and in vivo studies", *Eur J Pharm Biopharm,* vol. 79(1), pp. 3-14, 2011.

[23] C. Wang, Y. Gong, Y. Lin, J. Shen, D. A. Wang, "A novel gellan gel-based microcarrier for anchorage-dependent cell delivery", *Acta Biomater,* vol. 4(5), pp. 1226-1234, 2008.

[24] N. A. Silva, M. J. Cooke, R. Y. Tam, N. Sousa, A. J. Salgado, R. L. Reis, "The effects of peptide modified gellan gum and olfactory ensheathing glia cells on neural stem/progenitor cell fate", *Biomaterials*, vol. 33(27): pp. 6345-6354, 2012.

[25] B. N. Singh, L. T. Trombetta, K. H. Kim. "Biodegradation behavior of gellan gum in simulated colonic media", *Pham Dev Technol*, vol. 9, pp.399–407, 2004.