REFERENCES

Bonfiglio, M., and Jeter, W.S. (1972). Immunological responses to bone. Clinical Orthopaedics, 87, 19-27.

Bronwyn, M.K., Fiona, J.M., and John, H.M. (2000). Serum-free culture of rat postnatal and fetal brainstem neurons. Developmental Brain Research, 120, 199-210.

Chenite, A., Buschmann, M., Wang, D., Chaput, C., and Kandani, N. (2001). Rheological characterization of thermogelling chitosan/glycerol-phosphate solutions. Carbohydrate Polymers, 46, 39-47.

Chenite, A., Chaput, C., Wang, D., Combes, C., Buschmann, M.D., Hoemann, C.D., Leroux, J.C., Atkinson, B.L., Binette, F., and Selmani, A. (2000). Novel injectable neutral solutions of chitosan form biode-gradable gels in situ. Biomaterials, 21, 2155-2161.

Cho, T.J., Gerstenfeld, L.C., and Einhorn, T.A. (2002). Differential tem-poralexpression of members of the transforming growth factor beta superfamily during murine fracture healing. Journal of Bone and Mineral Research, 17, 513-520.

Choochottiros, C., Yoksan, R., and Chirachanchai, S. (2009). Amphiphilic chitosan nanospheres: Factors to control nanosphere formation and its consequent pH responsive performance. Polymer, 50, 1877–1886.

Cooke, F.W., (1992). Ceramics in orthopedic surgery. Clinical Orthopaedics, 276, 135–146.

Costantino, P.D., and Friedman, C.D. (1994). Synthetic bone graft substitutes. Otolaryngologic Clinics of North America, 27, 1037-1074.

Dang, Q.F., Jing, Q.Y., Li, J.J., Cheng, X.J., Liu, C.S., and Chen, X.G. (2011). Controlled gelation temperature, pore diameter and degradation of a highly porous chitosan-based hydrogel. Carbohydrate Polymers, 83, 171-178.

Devakara, R.E., William, R.T., Markus, O.H., and Georg, N.D. (2006). Mechanical conditions in the initial phase of bone healing. Clinical Biomechanics, 21, 646–655.

Du, C., Cui, F.Z., Feng, Q.L., Zhu, X.D., and Groot, K. (1998). Tissue response to nano-hydroxyapatite/collagen composite implants in marrow cavity. Journal of Biomedical Materials Research, 42, 540–548.

Fangkangwanwong, J., Yoksan, R., and Chirachanchai, S. (2006). Chitosan gel formation via the chitosaneepichlorohydrin adduct and its subsequent mineralization with hydroxyapatite. Polymer, 47, 6438-6445.

Focher, B., Massoli, A., Torri, G., Gervasini, A., and Morazzoni, F. (1986). Macromolecular Chemistry and Physics, 87, 2609.

Freesia, L.H., and Warren, H.E. (1972). Incorporation of glucosamine into maturing bone marrow granulocytes. Biochimica et Biophysica Acta, 273, 157-164.

Goldberg, V.M., and Stevenson, S. (1987). Natural history of autografts and allografts. Clinical Orthopaedics, 225, 7-16.

Guibal, E. (2004). Interaction of metal ions with chtosan based sorbents: a review. Separation and Purification Technology, 38, 43-74.

Guillen, G. P., and Madroñero, A.C. (1985). Enhancement of bone healing by an exogenous magnetic field and the magnetic vaccine. Journal of Biomedical Engineering, 7, 157-160.

Guobao, W., and Peter, X.M. (2004). Structure and properties of nano-hydroxyapatite/polymer composite scaffolds for bone tissue engineering. Biomaterials, 25, 4749-4757.

Hockin, H.K., Michael, D., and Carl, G. (2008). Injectable and strong nano-apatite scaffolds for cell/growth factor delivery and bone regeneration. Dental Materials, 24, 1212-1222.

Holme, K.R., and Perlin, A.S. (1977). Chitosan *N*-sulfate. A water-soluble polyelectrolyte. Carbohydrate Research, 302, 173.

Hulstyn, M., Fadale, P.D., Abate, J., and Walsh, W.R. (1993). Biomechanical evaluation of interference screw fixation in a bovine patellar bone-tendon-bone autograft complex for anterior cruciate ligament reconstruction. The Journal of Arthroscopic & Related Surgery, 9, 417-424.

Hunter, W.L., Burt, H.M., and Machan, L. (1997). Local delivery of chemo-therapy: a supplement to existing cancer treatments a case for surgical pastes and coated stents. Advanced Drug Delivery Reviews, 26, 199–207.

Ilium, L. (1998). Chitosan and its use as a pharmaceutical excipient. Pharmaceutical Research, 15, 1326-1331.

Ishaug, S.L., Crane, G.M., Miller, M.J., Yasko, A.W., Yaszemski, M.J., and Mikos, A.G. (1997). Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. Journal of Biomedical Materials Research, 36, 17–28.

Jager, T., Journeau, P., Dautel, G., Barbary, S., Haumont, T., and Lascombes, P. (2010). Is combining massive bone allograft with free vascularized fibular flap the children's reconstruction answer to lower limb defects following bone tumour resection? Orthopaedics & Traumatology: Surgery & Research, 96, 340-347.

Jayakumar, R., Prabaharan, M., Nair, S.V., Tokura, S., Tamura, N., and Selvamurugan, A. (2010). Novel carboxymethyl derivatives of chitin and chitosan materials and their biomedical applications. Progress in Materials Science, 55, 675-709.

Kaufman, J.J., and Chiabrera, A.E. (1997). Ultrasonic Bone Assessment Apparatus and Method. United States Patent, 5, 651-363.

Kendra, D.F., and Hadwiger, L.A. (1984). Characterization of the smallest chitosan oligomer that is maximally antifungal to Fusarium soloni and elicits pisatin formation in Pisum sativum. Experimental Mycology, 8, 276.

Le Geros, R.Z. (2002). Properties of osteoconductive biomaterials: calcium phosphates. Clinical Orthopaedics, 395, 81–98.

Lertwattanaseri, T., Ichikawa, N., Mizoguchi, T., Tanaka, Y., and Chirachan-chai, S. (2009). Microwave technique for efficient deacetylation of chitin nano-whiskers to a chitosan nanoscaffold. Carbohydrate Re-search, 344, 331-335.

Li, S.H., De Wijn, J.R., Layrolle, P., and de Groot K. (2002). Synthesis of macroporous hydroxyapatite scaffolds for bone tissue engineering. Journal of Biomedical Materials Research, 61, 109–120.

Li, X., Kong, X., Wang, X., Shi, S., Guo, G., Luo, F., Zhao, X., Wei, Y., and Qian, Z. (2010). Gel-sol-gel thermo-gelation behavior study of chitosan inorganic phosphate solutions. European Journal of Phar-maceutics and Biopharmaceutics, 75, 388-392.

Lin, S., Walls, H.J., Kennedy, S.B., and Weish, E.R. (2003). Reaction kinetics and gel properties of blocked diisocyinate crosslinked chitosan hydrogels. Carbohydrate Polymers, 54, 193-199.

Ma, PX., Zhang, R., and Xiao, G. (2001). Engineering new bone tissue in vitro on highly porous poly(alpha-hydroxyl acids)/ hydroxyapatite com-posite scaffolds. Journal of Biomed Mater Research, 54, 284–293.

Malmsten, M., and Lindman, B. (1992). Self-assembly in aqueous block copolymer solutions. Macromolecules, 25, 5440-5445.

Manjubala, I., Scheler, S., Jörf, B., and Klaus, D. (2006). Mineralization of chitosan scaffolds with nano-apatite formation by double diffusion technique. Acta Biomaterialia, 2, 75-84.

Martin, J.G., Thakur, R.S., David, A. W., and Ryan F. D. (2010). Poly(caprolactonetriol) as plasticizer agent for cellulose acetate films: influence of the preparation procedure and plasticizer content on the physico-chemical properties. International Journal of Pharmacy and Pharmaceutical Sciences, 406, 91–98.

Mihammad, R.K. (2010). Determination of the degree of *N*-acetylation for chitin and chitosan by various NMR spectroscopy techniques: A review. Carbohydrate Polymers, 79, 801-810.

Mirko, X.W., Janelle, C.M., Jolanta, K., and Jorg T. (2009). Studies on acetylation patterns of different chitosan preparations. Carbohydrate Polymers, 78, 678-684.

Muzzarelli, R.A.A. (1996). Chitosan-based dietary foods. Carbohydrate Polymers, 29, 309-316.

Nishi, N., Maekita, Y., Nishimaru, S., Hasegawa, O., and Tokura, S. (1987). Highly phosphorylated derivatives of chitin, partially deacetylated chitin and chitosan as new functional polymers: metal binding property of the insolubilized materials. International Journal of Biological Macromolecules, 9, 109-114.

Park, H., and Park, K. (1996). Oral vaccination with alginate microsphere systems. Journal of Controlled Release, 39, 209-220.

Paul, J.F., and John, R.J. (1943). Investigation of solute permeation across hydrogels composed of poly(methyl vinyl ether-co-maleic acid) and poly(ethylene glycol). Journal of Chemical Physics, 11, 512–517.

Phongying, S., Aiba, S., and Chirachanchai, S. (2006). Direct chitosan nanoscaffold formation via chitin whiskers. Polymer, 48, 393-400.

Poumarat, G., and Squire, P. (1993). Comparison of mechanical properties of human, bovine bone and a new processed bone xenograft. Biomaterials, 14, 337-340.

Rao, S.B., and Sharma, C.P. (1992). Use of chitosan as a biomaterial: Studies on its safety and hemostatic potential. Journal of Biomedical Materials Research, 34, 21-28.

Ramazani-Harandi, M.J., Zohuriaan-Mehr M.J., Yousefi, A.A., Ershad Langroudi, A., and Kabiri, K. (2006). Rheological determination of the swollen gel strength of superabsorbent polymer hydrogels. Polymer Testing, 25, 470-474.

Rho, J.Y., Kuhn-Spearing, L., and Zioupos, P. (1998). Mechanical properties and the hierarchical structure of bone. Medical Engineering & Physics, 20, 92–102.

Richardson, S.C.W., Kolbe, H.V.J., and Ducan, R. (1999). Potential of low molecular mass chitosan as a DNA delivery system: biocompatibility, body distribution and ability to complex and protect DNA. International Journal of Pharmaceutics, 178, 231-243.

Rinaudo, M. (2006). Chitin and chitosan: properties and applications. Pro-gress in Polymer Science, 31, 603-632.

Sakou, T. (1998). Bone morphogenetic proteins: from basic studies toclinical approaches. Bone, 22, 591-603.

Shimizu, Y., Kono, K., Kim, S., and Takashi, T. (1995). Effect of added metal ions on the interaction of chitin and partially deacetylated chitin with an azo dye carrying hydroxyl groups. Journal of Appied Polymers Science, 55, 255-261.

Silverman, R.P., Passaretti, D., Huang, W., Randolph, M.A., and Yaremchuk, M.J. (1999). Injectable tissue-engineered cartilage using a fibrin glue polymer. Journal of Plastic and Reconstructive Surgery and Hand Surgery, 103, 1809–1818.

Sims, C.D., Butler, P., Casanova, R., Lee, B.T., Randolph, M.A., Lee, A., and Vacanti, C.A. (1996). Injectable cartilage using polyethylene oxide polymer substrates. Journal of Plastic and Reconstructive Surgery and Hand Surgery, 95, 843–850.

Sunil, K.B., and Surinderpal, S. (2006). Analysis of swelling behaviour of poly(methacrylamide-co-methacrylic acid) hydrogels and effect of synthesis conditions on water uptake. Reactive and Functional Polymers, 66, 431–440.

Suzuki, S. Watanabe, T., Mikami, T., and Suzuki, M. (1986). Chitin in Nature and Technology, Plenum Press, New York and London.

Tang, Y., Wang, X., Li, Y., Lei, M., Du, Y., Kennedy, J.F., and Knill, C.J. (2010). Production and characterization of novel injectable chitosan/ methylcellulose/ salt blend hydrogels with potential application ad tissue engineering scaffolds. Carbohydrate Polymers, 82, 833-841.

Thakur, R.R.S., Woolfson, A.D., and Donnelly, R.F. (2010). Investigation of solute permeation across hydrogels composed of poly(methyl vinyl ether-co-maleic acid) and poly(ethylene glycol). Journal of Pharmacy and Pharmacology, 62, 829–837.

Thomson, R.C., Yaszemski, M.J., Powers, J.M., and Mikos, A.G. (1998). Hydroxyapatite fiber reinforced poly(alpha-hydroxy ester) foams for bone regene-ration. Biomaterials, 19, 1935–1943.

Tsai, M.L., Chang, H.C., Yu, H.C., Lin, Y.S., and Tsai Y.D. (2011). Effect of chitosan characteristics and solution conditions on gelation temperatures of chitosan/ 2-glycerophosphate/ nanosilver hydrogels. Carbohydrate Polymers, 84, 1337-1343.

Tuncer, C., Simin, K., and GöKhan, D. (2006). Network Parameters and Volume Phase Transition Behavior of Poly(N-isopropylacrylamide) Hydrogels. Journal of Applied Polymer Science, 101, 1756–1762.

Visscher, G.E., Robison, R.L., Maulding, H.V., Fong, J.W., Pearson, J.E., and Argentieri, G.J. (1985). Biodegradation of and tissue reaction to 50:50 poly-

(*DL*-lactide-co-glycolide) microcapsules. Journal of Biomedical Materials Research, 19, 349–65.

Wang, J., and Wu,W. (2005). Swelling behaviors, tensile properties and thermodynamic studies of water sorption of 2-hydroxyethyl methacrylate/ epoxy methacrylate copolymeric hydrogels. European Polymer Journal, 41, 1143–1151.

Wakitani, S., Kimura, T., and Hirooka, A. (1989). Repair of rabbit articular surfaces with allograft chondrocytes embedded in collagen gel. The Journal of Bone and Joint Surgery, 71, 74–80.

Webster, T.J., Ergun, C., Doremus, R.H., Siegel, R.W., and Bizios, R. (2000). Specific proteins mediate enhanced osteoblast adhesion on nanophase ceramics. Journal of Biomedical Materials Research, 51, 475–483.

Yamamoto, H., and Amaike, M. (1997). Biodegradation of cross-linked chitosan gels by a microorganism. Macromolecules, 30, 3936-3937.

Zhang, C., Ping, Q., Zhang, H., and Shen, J. (2003). Synthesis and characterization of water-soluble *O*-succinyl-chitosan. European Polymer Journal, 39, 1629–1634.

Zhang, R., and Ma, P.X. (1999). Porouspoly(*l*-lactic acid)/ apatite composites created by biomimetic process. Journal of Biomedical Materials Research, 45, 285–293.

Zhang, R., and Ma, P.X., (1999). Poly(alpha-hydroxyl acids)/ hydroxyapatite porous composites for bone tissue engineering. I. Preparation and morphology. Journal of Biomedical Materials Research, 44, 446–455.

Zhou, J., Lee, M.J., Jiang, P., Henderson, S., and Lee, D.G. (2010). Reduction in postsurigical adhesion formation after cardiac surgery by application of *N,O*-carboxymethyl chitosan. The Journal of Thoracic and Cardiovascular Surgery, 140, 801-806.

APPENDICES

Appendix A DNA Assay

Objective

Fluorescent measurement used to evaluate cell number by converted from calibration curve of known cell number.

Principle

The fluorescent bisbenzimide (Hoechst 33258) dry is relatively selective for dsDNA and does not show fluorescent enhancement in the presence of either protein or RNA. The dry, weakly fluorescents itself in solution, binds specifically to the A-T base pairs in dsDNA resulting in an increase in fluorescence and a shit in the emission maximum from 500 to 460 nm. The use of Hoechst 33258 in conjunction with the fluorescence microplate reader offers high specificity, as well as high sensitivity for dsDNA quantitation.

Materials

1. SSC solution (20X)

   17.999 g NaCl + 8.823 g $Na_3Citrate.\ 2H_2O$

   Add DI water upto 100 ml
2. SDS lysis buffer

   SDS 20 mg + (5 ml of 20X SSC + 95 ml DDW)
3. Hoechst 33258 solution (1 mg/ml) = Bisbenzimide H33258 Fluorochrome Trihydrochloride in solution (1 mg/ml), ($C_{25}H_{24}N_6O.3HCl$, MW = 533.88, store at -20°C)
4. DI water
5. 1-ml vials
6. 50-ml centrifugal tube
7. Multi-channel autopipette and tips
8. 96-well bank plate

Method

Preparation of cell lysates from the samples (cell cultured on film or scaffold)

1. Freeze the cell samples (cell cultured on film or scaffold) and cell standard (10^6 cells/1 vial) at -80°C and thaw at room temperature several cycles to break cell membrane

2. For the case of cells/scaffold construct, mince the samples into small pieces

3. Add 1 ml SDS lysis buffer to each samples and cell standard, sonicate and incubate at 37°C for 1 h to break cell membrane completely and obtain cell lysate

Measurement of DNA

4. Serial dilute standard cell solution with SDS lysis buffer to prepare various concentrations of cell standards

5. Pipette 100 μm of cell lysates (samples prepared from step 3), cell standards (prepared from step 4) and DI (blank) into 96-well black plate

6. Thaw Hoechst 33258 at room temperature and prepare Hoechst 33258 solution Hoechst (20 μl) + DI (19 ml) + SSC (1 ml)

7. Add 100 μl of the Hoechst 33258 solution (prepared from step 6) into each well using muti-channel autopipette

8. Immediately measure the fluorescent intensity of solution at 355 nm (Excitation) and 460 nm (Emission) using fluorescent microplate reader

Hint

1. Hoechst 33258 solution must always be kept at -20°C in dark condition to preserve its activity, then it should be warmed just before use.

2. Multi-channel autopipette must be used in order to shorten the time. Also, addition of Hoechst 33258 solution and cell lysates should be done in the same order in order to control the reaction time.

3. Steps 6-8 must be done in dark to preserve the fluorescent intensity of dry solution.

4. The fluorescent intensity of solution must be measured immediately after adding the Hoechst 33258 solution because intensity of fluorescence reduces with time.

Appendix B Drying Technique (Freeze-dry)

In generally after complete gel process, these are many techniques to convert wet gels to aerogels such as, supercritical drying technique, drying control chemical additives and ambient-pressure drying technique (Fricke *et al.*, 1997). Recently, dehydration by lyophilizing is accepted as a practical technique.

This technique can be divided into three stages: freezing, primary drying and secondary drying.

a. Freezing

The freezing process is done by placing the material in a freeze-drying flask ting the flask in a bath, called a shell freezer. It is important to freeze the material at temperature below the eutectic point of material because this point ensures that sublimation rather than melting will occur in the following steps.

b. Primary Drying

In this phase, the pressure is lowered and enough heat is supplied to the material for water to sublimate. This phase may be shown, because if too much heat is added the material's structure could be altered.

c. Secondary Drying

In this phase, the secondary drying phase is possible drying when the water molecules that are absorbed during the freezing process are sublimed. In this drying process is governed by the material's adsorption isotherms. Temperature is raised even higher than in primary drying phase to break any physio-chemical interactions that have formed between the water molecules and the frozen material (http: //en.wiki-pedia.org/wiki/Freeze_drying).

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