Zirconia Macrocapsules

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Bioceramic Macrocapsules for Cell Immunoisolation**

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Bone damage and diseases cause severe health problems to patients whose healing often requires surgical interventions. Conventional graftings, such as autographing, allographing, and xenographing all have inherent disadvantages.[1] Artificial implants made of biomaterials are alternatives for bone treatments. However, such treatments require the implant materials to be highly biocompatible to facilitate bone healing. For instance, porous biomaterials exhibit the potential for bone reconstruction and regeneration, but the control of porous structures and the requirements for osteoconduction and osteoinduction pose many challenges.[2] Alternatively, seeding cells in engineered scaffolds is a promising approach for enhancing bone reconstruction and remodeling. However, a high risk of infection owing to immune rejection has hindered its progress.[3] Bioceramic capsules may provide immunoisolation to circumvent this problem. Osteoinductive bone morphogenic proteins (BMPs) produced in human bodies can be used to stimulate bone formation/healing.[4] However, BMPs are very expensive and liable to be degraded around the wound sites. Thus, it is ideal to use cells to continuously generate BMPs in situ and simultaneously restrict the passage of large immune-system molecules and antigens. To meet this challenge, we propose that osteoblast cells or osteogenic stem cells be modified by gene therapy to yield BMPs. These conditioned cells are then encapsulated and integrated with artificial porous bodies for final implantation. We expect that the cells would secrete BMPs for bone formation and remodeling without the risk of immune rejection. Herein, we report the development of porous zirconia ceramic macrocapsules with unique properties that are suitable as immunoisolation devices for bone reconstruction.

Herein, ZrO_2 macrocapsules with an asymetric structure were prepared through the combination of phase-inversion and sintering techniques. The two parts (tubular body and the sealing end) of the capsule precursors have a similar material composition and were intergrated very well after sintering as they experienced similar polymer decomposition processes and volume shrinkage. As illustrated in Figure 1a, there is no discrimination between the sealing part and tubular fiber. Figure 1b shows the morphology of the capsule near the opening end. The outer diameter and inner diameter measured from Figure 1b are 1.5 mm and 1.0 mm, respectively. Figure 1c and Figure 1d show the microporous structures of the outside and inside surface of the capsule. After sintering, the zirconia grain size was increased from 80 nm to 300 nm, whereas the length of the capsule (10–14 mm) suffered a length shrinkage of about 20%. Contact-angle measurements confirm that the prepared zirconia capsule is highly hydrophilic. The chamber volume is about 10 μL. Such physical dimensions of the capsule can be fine-tuned to suit different practical applications.

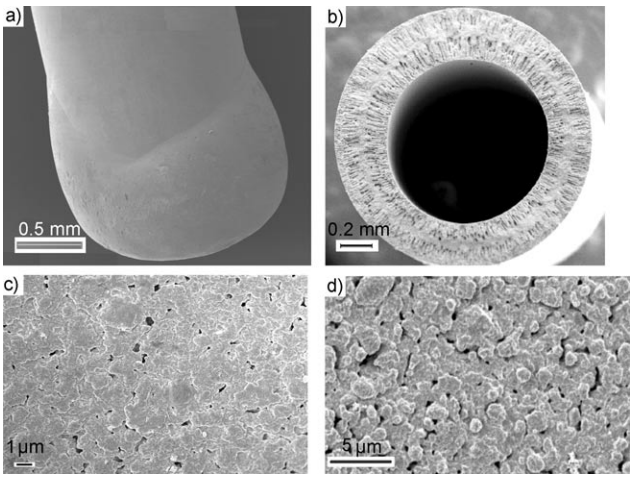

Figure 1. Typical scanning electron microscopy (SEM) images of zirconia macrocapsules: a) part of the capsule near the closing end; b) the open side; c) the outside surface; and d) the inside surface.

Without further surface modification, the prepared zirconia ceramic capsules were tested for cell response. Osteoblast MC3T3-E1 cells were cultured on the capsular outer surface. The seeding situation on the ceramic surface was then examined by SEM after incubation in vitro for a series of prescribed times. Starting from the early attachment, cells experienced successive stages of proliferation, migration, and mineralization. At day 14, cells were populating the entire capsule outer surface and multilayers of bone nodules were observed (Figure 2a). After mineralization, many microspheres of minerals were distributed among extracellular space (Figure 2b). All these cell interactions on the capsule

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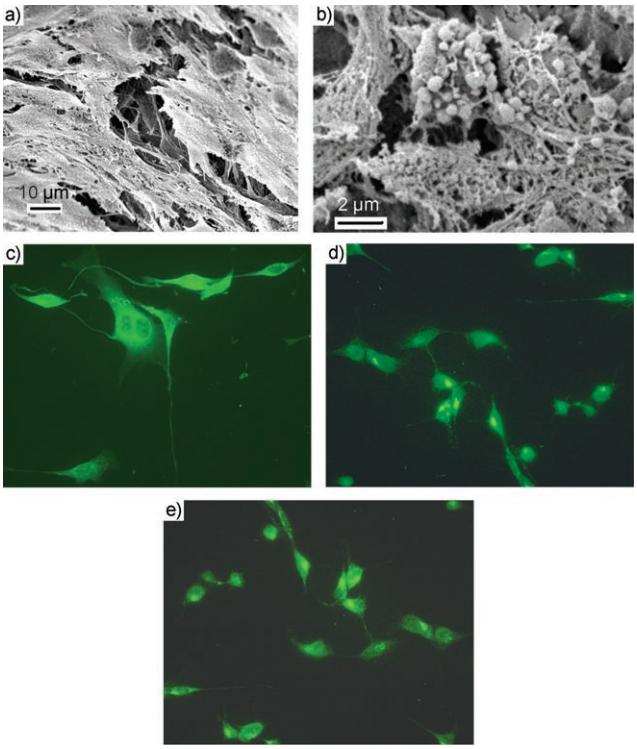

Figure 2. SEM images of multilayer MC3T3-E1 cells (a) and mineralized nodules (b) attached to the capsule outside surface after incubation for two weeks. c–e) Immunofluorescence results of differentiated MC3T3-E1 cells. Collagen type I (c), osteopontin (d), and osteocalcin (e) proteins were well expressed by cells after contacting and co-incubating with ZrO_2 macrocapsules.

outer surface indicate the good biocompatibility of capsules or at least that the capsules did not show any detrimental effect on the osteoblasts because bone cells do not proliferate on a nonideal surface. How to enhance the cell interactions with biomaterial surfaces is the focus of many recent research endeavors. Previous studies indicated that osteoblast adhesion is independent of ceramic surface chemistry and material phase but strongly dependent on the ceramic surface topography.[5] For example, enhanced osteoblast adhesion on ceramic surfaces can be achieved if bioceramics are made from nanophase alumina or titania. In agreement with this, the current capsules were also made from nanosized ceramic powders and may provide a good microenvironment to promote cell-substrate interactions.

We have further observed that the zirconia capsule can also promote cell differentiation. MC3T3-E1 cells, cocultured with ZrO_2 capsules and then detached from the capsular surface, are highly viable. Bone nodules were observed at day 2 in a petri dish even before the cells formed a confluent monolayer. These nodules increased in size during the following days and became denser and more opaque. A few differentiation proteins were generated within the cells as identified by the uniform immunofluorescence intensity. As presented in Figure 2c, collagen type I, one of early markers expressed by proliferative osteoblasts,[6] was quite uniformly distributed in the cytoplasm. Osteopontin (Figure 2d), a marker of MC3T3-E1 cell differentiation in the intermediate stage, indicates the matrix formation and maturation. Another detected protein, osteocalcin (Figure 2e), shows the progressing of the matrix mineralization in the cells.[7]

Zirconia ceramic material is well known for its high mechanical strength. As reported previously, the three-point bending strength of a ZrO_2 hollow fiber is 220 MPa, which is four times that of alumina hollow fibers prepared under similar conditions.[8,9] Moreover, the short ceramic capsules are more difficult to break and therefore more appropriate for applications in hard-tissue engineering. The average pore size of the capsule is 0.5 μm as characterized by gas-permeation techniques that use nitrogen as the test gas.[10] Capsules with such a pore structure allow the free permeation of most biomolecules (nutrients and proteins) but the passage of large immune molecules and cells is restricted, thus the objectives of cell immunoisolation can be achieved. Cells from one species can be transplanted into a discordant host species to continuously deliver the secreted biomolecules without the risk of immune rejection. In the present study, we injected MC-3T3-E1 cells into the capsular chamber and sealed the open end with sterile zinc phosphate cement, a good sealing material that is used by dentists for tooth disease treatment and does not exhibit toxicity towards cells.

The encapsulated MC3T3-E1 cells were incubated in vitro for one week and the cell viability was carefully examined by SEM. Bacterial colonization was not found during the whole incubation process for all samples. Again, cells initially attached onto the capsule inner surface (Figure 3a) and then continuously proliferated, differentiated, and migrated with increasing incubation time. As shown in Figure 3b, cells

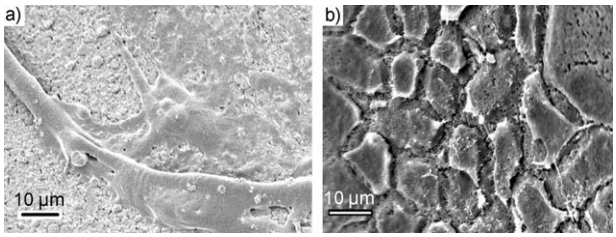

Figure 3. SEM images of encapsulated cells by ZrO_2 macrocapsules after incubation for 4 days (a) and 7 days (b).

populated the entire inner surface of the capsule at day 7. Such well-maintained growth (or viability) of encapsulated cells within the capsule indicates that critical nutrient molecules, gases (O_2 and CO_2), and necessary biomolecules (fatty acids, amino acids, and base pairs) can freely permeate into the encapsulated space to nourish the confined cells during the culturing process. However, these confined cells within the capsule cannot permeate through the chamber to the outside environment owing to their relatively big size (roughly 50–100 μm versus 0.5 μm of the membrane pore size). Successful isolation and growth of MC3T3-E1 cells is an absolute requirement for future genetically modified bone cell lines to be considered clinically.

Similarly, the biomolecules secreted by MC3T3-E1 cells in the capsular chamber can permeate freely into the surround-

ings, as demonstrated by the easy release of bovine serum albumin (BSA) with a molecular weight of 66 kDa (corresponding to the average BMP molecular weight) under ambient conditions. The capsule was filled with BSA (4 μL, 20 μg μL^{-1}) by a microinjector and sealed with zinc phosphate cement. As shown in Figure 4, about 84% of the BSA

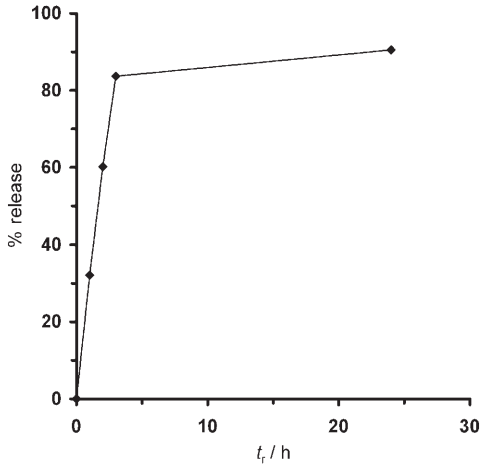

Figure 4. The release profile of BSA from ZrO_2 macrocapsules into PBS buffer solution (the initial quantity of encapsulated protein was 80 μg; t_r = release time).

diffused from the capsular chamber in the first three hours, with an average diffusion rate of 22.3 μg h^{-1}, indicating the low resistance for BSA permeation. This can be easily understood as the pore size of the capsular wall is 0.5 μm, much larger than a molecule of BSA, which is around 10–15 nm in size. However, a slower release rate (7% in total) was noted in the following 21 h. This can probably be attributed to the diffusion of BSA that was trapped inside the smaller pores. The application of polymeric materials in protein delivery has been often frustrated by protein denaturizing and fouling owing to the hydrophobic nature of the membrane surface.[11] By contrast, zirconia ceramic capsules developed in this work are intrinsically hydrophilic and show low sorptivity for the BSA protein, do not denature and foul proteins, and not undergo the flux decay in the presence of such proteins.

In conclusion, the tailor-made zirconia macrocapsules with a chamber volume of 10 μL are biocompatible and friendly to MC3T3-E1 cells and moreover can facilitate osteoblast cell differentiation. Cells encapsulated within such capsular chambers are viable and grow well by absorbing the nutrients permeated through the capsule micropores from the outside environment. BSA, an example of a protein, was released from the capsules to the surrounding environment without any resistance and fouling. Our experimental results indicate that the developed zirconia capsule is novel and promising as immunoisolation medical templates for bone-tissue engineering and micro-bioreactors for biomolecule delivery to specific sites.

Experimental Section

Zirconia macrocapsules: Y_2O_3 (3%) partially stabilized ZrO_2 powder with a particle size of 80 nm (Shandong, China), polyethersulfone (PESf; Radel A-300, Ameco Performance), and *N*-methyl-2-pyrrolidone (NMP; (Synthesis Grade, Merck) were used as raw materials to prepare the capsule precursor. The mixture containing 40% (wt) NMP, 10% PESF, and 50% ZrO_2 was thoroughly stirred to ensure uniform distribution of the particles. Hollow fiber extrusion was carried out through a tube-in-orifice spinneret with details described elsewhere.[8] The dried ZrO_2 hollow-fiber precursor was cut into short fibers with a length around 15 mm. These short hollow fibers were capped at one end with the same mixture of materials that were initially used for fiber spinning. Briefly, the mixture was spread on a smooth glass slide into a 1.5-mm-thick layer on which the short fiber was then vertically placed. The sealed fibers were immediately transferred into water to complete the phase inversion. The capped short hollow fibers were dried and subsequently sintered at 1400 °C for 10 h to form ZrO_2 ceramic capsules.

Cell culture and differentiation: Mouse MC-3T3-E1 osteoblast cells were obtained from ATCC, USA. The culture medium was alpha minimum essential medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were incubated at 37 °C in 5% CO_2/95% air environment and further subcultured by Trypsin–EDTA at 70–80% confluence. Cells on the ZrO_2 surface were perfused with phosphate-buffered saline solution (PBS), fixed by 2% gluteraldehyde, and then dehydrated in ethanol with ascending gradient concentration from 35% to 100%. A Samdri 780A critical point dryer was employed to dry the constructs before coating for SEM observation.

Several antibody proteins were selected for cell-differentiation tests. Rabbit anti-mouse collagen type I (Calbiochem, CA), osteopontin (Assay Designs, Michigan), and osteocalcin (Takara, Japan) affinity-purified polyclonal antibodies were used as primary antibodies. Fluorescein-linked anti-rabbit antibody (Amersham Biosciences, NJ) was chosen as the secondary antibody. Cells detached from the capsule surface were firstly incubated with primary antibodies overnight at 4 °C. After that, cells were incubated with the secondary antibodies followed by PBS washing. A Zeiss immunofluorescence microscope was used to take the photographs. Controls for each antibody consisted of probing with the secondary antibody in the absence of the primary antibody.

Cell encapsulation and protein release: Capsules were pre-equilibrated with the culture medium for 1 day. To evaluate the cell viability in the capsules, growth medium (2.5 μL) containing cells at a density of 10^6 cells mL^{-1} was loaded inside the capsule chamber by a Hamilton microinjector (Stoelting, USA), followed by sealing with HY-Bond zinc phosphate cement (SHOFU INC, Japan). After incubation for a prescribed time period, 3T3 cell morphology was examined by using SEM.

BSA (Sigma, USA) with a molecular weight of 66 kDa was used for investigating the protein-release profile from ZrO_2 macrocapsules. The permeated protein amount was determined by the Bradford Assay reagent (Bio-Rad, USA). Typically, ZrO_2 macrocapsules were filled with BSA (4 μL , 20 μg μL^{-1}) by microinjector and sealed with zinc phosphate. The capsules were immersed in PBS (500 μL) with 10 μL of the PBS solution withdrawn at the various time points. Absorbance at 620 nm on a Biotrak II microplate reader (Amersham Biosciences, USA) was recorded to determine the BSA concentration.

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[1] a) A. J. Yaszemski, R. G. Payne, W. C. Hayes, R. Langer, A. G. Mikos, *Biomaterials* **1996**, *17*, 175 – 185; b) J. Lane, H. Sandhu, *Orthop. Clin. North Am.* **1987**, *18*, 213 – 219; c) W. F. Enneking, E. R. Mindell, *J. Bone Jt. Surg. Ser. Am.* **1991**, *73*, 1123 – 1142.
[2] a) J. Goshima, V. M. Goldberg, A. I. Caplan, *Biomaterials* **1991**, *12*, 253 – 258; b) T. Yoshikawa, H. Ohgushi, H. Nakajima, E. Yamada, K. Ichijima, S. Tamai, T. Ohta, *Transplantation* **2000**, *69*, 128 – 134; c) R. J. Dekker, J. D. de Bruijn, I. van den Brink, Y. P. Bovell, P. Layrolle, C. A. van Blitterswijk, *J. Mater. Sci. Mater. Med.* **1998**, *9*, 859 – 863; d) J. M. Wozney, R. H. Li, *Nat. Biotechnol.* **2003**, *21*, 506 – 508.
[3] J. E. Babensee, J. M. Anderson, L. V. McIntire, A. G. Mikos, *Adv. Drug Delivery Rev.* **1998**, *33*, 111 – 139.
[4] a) M. Hoshino, T. Egi, H. Terai, T. Namikawa, K. Takaoka, *Biomaterials* **2006**, *27*, 4934 – 4930; b) S. R. Winn, H. Uludag, J. O. Hollinger, *Adv. Drug Delivery Rev.* **1998**, *31*, 303 – 318.
[5] a) T. J. Webster, R. W. Siegel, R. Bizios, *Nanostruct. Mater.* **1999**, *12*, 983 – 986; b) T. J. Webster, R. W. Siegel, R. Bizios, *Biomaterials* **1999**, *20*, 1221 – 1227; c) T. J. Webster, S. L. Schadler, R. W. Siegel, *Tissue Eng.* **2001**, *7*, 291 – 301; d) T. J. Webster, C. Ergun, R. H. Doremus, *Biomaterials* **2001**, *22*, 1327 – 1333.
[6] P. Ducy, M. Starbuck, M. Priemel, J. Shen, G. Pinero, V. Geoffroy, M. Amling, G. A. Karsenty, *Genes Dev.* **1999**, *13*, 1025 – 1036.
[7] G. A. Candeliere, F. Liu, J. E. Aubin, *Bone* **2001**, *28*, 351 – 361.
[8] L. Liu, S. Gao, Y. Yu, R. Wang, D. Liang, S. Liu, *J. Membr. Sci.* **2006**, *280*, 375 – 382.
[9] S. Liu, K. Li, *J. Membr. Sci.* **2003**, *218*, 269 – 277.
[10] a) X. Tan, S. Liu, K. Li, *J. Membr. Sci.* **2001**, *188*, 87 – 95; b) H. Yasuda, J. T. Tsai, T. J. Webster, R. W. Siegel, R. Bizios, *J. Appl. Polym. Sci.* **1974**, *18*, 805 – 819; c) K. Li, K. J. Feng, *AICHE J.* **1999**, *45*, 1211 – 1219.
[11] a) R. H. Li, *Adv. Drug Delivery Rev.* **1998**, *33*, 87 – 109; b) A. S. Michaels, *Desalination* **1990**, *77*, 5 – 34; c) T. Knoell, J. Safarik, T. Cormack, R. Riley, S. W. Lin, H. Ridgway, *J. Membr. Sci.* **1999**, *157*, 117 – 138.

