J|A|C|S
COMMUNICATIONS

Published on Web 10/11/2006

Assembly of Three-Dimensional Polymeric Constructs Containing Cells/Biomolecules Using Carbon Dioxide

Yong Yang,[†,‡] Yubing Xie,[†] Xihai Kang,[†,§] L. James Lee,*[,†,‡] and Douglas A. Kniss[§]

Nanoscale Science and Engineering Center for Affordable Nanoengineering of Polymeric Biomedical Devices (NSEC-CANPBD), Department of Chemical and Biomolecular Engineering, Department of Obstetrics & Gynecology, and Department of Biomedical Engineering, The Ohio State University, Columbus, Ohio 43210

Received August 24, 2006; E-mail: lee.31@osu.edu

The demand for high-precision polymeric miniature devices, particularly for biomedical applications, is growing rapidly. Biologically permissive processing and assembly techniques that meet strict design constraints (noncontaminating, nondeforming, low temperature) are necessary. For example, polymeric nanoporous gate films can be used to seal protein- and/or cell-containing reservoirs to achieve controlled drug release and/or immunoisolation.[1] In tissue engineering, it is critical to create three-dimensional (3-D) scaffolds with predefined structures to control the spatial organization of cells and to provide the necessary cues to recapitulate the developmental processes in tissue differentiation and morphogenesis.[2−5] Of vital concern is the production and assembly of 3-D constructs in the presence of cells and/or biomolecules. Of equal concern is preserving the original micro/nanosized structures after processing.

Conventional polymer processing methods, using either organic solvents or high temperatures, are detrimental to solvent- or thermal-sensitive biomolecules and cells[6] and tend to deform microstructures.[7] In this Communication, we present a simple and versatile approach to assembling polymer-based 3-D constructs containing cells/biomolecules using low-pressure CO_2. This method highlights the ability to construct multiple cell-scaffold constructs into a predesigned tissue complex. Pluripotent embryonic stem cells (ESCs) and bone marrow derived mesenchymal stem cells (MSCs), which hold great promise as unlimited cell sources in tissue engineering and cell therapy, were used to demonstrate the feasibility of this assembly approach.

The possibility of using supercritical CO_2 ($scCO_2$) as a solvent for polymer processing has been extensively explored as it is an unregulated and noncontaminating processing aid. Supercritical CO_2 has been utilized to incorporate biomolecules, for example, proteins and DNAs, into polymer matrices for drug delivery and tissue engineering.[6,8,9] Recently, $scCO_2$ has been used to process some mammalian cells into polymer composite structures as well.[10]

Unlike the fusion of polymers using $scCO_2$ in previous studies,[11−13] we found that low-pressure CO_2 can enhance polymer chain mobility as well as reduce the glass transition temperature (T_g) of polymers at the nanoscale. The T_g near the polymer surface is lower than the bulk value. The presence of CO_2 not only depresses the bulk T_g but also greatly reduces the T_g at the surface and broadens the rubbery layer at the same temperature. These effects increase with CO_2 pressure. For instance, a CO_2 pressure as low as 0.69 MPa can decrease the bulk T_g of poly (DL-lactide-co-glycolide) (PLGA) from 48.3 °C to 40.9 °C and produce a surface mobile layer with a depth of 18 nm at 37 °C, making it possible to fuse polymeric micro-/nanostructures.[14,15]

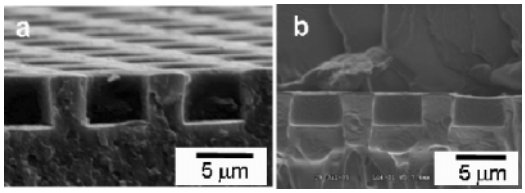

Figure 1. SEM micrographs of the cross-section of PLGA microstructure (a) before and (b) after fused at 37 °C and 0.69 MPa CO_2 pressure in PBS.

To explore the abilities of CO_2-assisted fusion of polymeric microstructures in an aqueous environment, PLGA layers were patterned with features as small as 3.9 μm (Figure 1a). Several patterned PLGA layers were stacked and held in a container filled with phosphate-buffered saline (PBS) under a compressive pressure of 69 kPa, which ensured intimate contact between these layers. The system was then placed in a pressure vessel at 37 °C. After being exposed to CO_2 at 0.69 MPa for 15 min, the multiple PLGA layers were fused into a 3-D construct while preserving the original microstructures (Figure 1b). This indicates that CO_2 can diffuse into fluids, such as water, buffer solution, and cultivation media, and fused polymeric structures.

This technique has great potential for cells and/or biomolecules to be integrated into the fabrication and assembly processes. We first determine whether cells can survive in the context of a CO_2-assisted assembly. Human MSCs (hMSCs) were placed in a pressure vessel and CO_2 was injected at a rate of 0.28 MPa/min and maintained at 1.38 MPa for 30 min. After a slow and constant depressurization process (0.05 MPa/min), the hMSCs remained viable and continued attachment to the substrate (comparing parts a and b of Figure 2). This indicates that greater than 99% hMSCs were viable following CO_2 treatment even when the pH dropped from 7.4 to 6.5 during pressurization (the process varied from 15 min to 2 h). The slow and constant depressurization reduced, even eliminated, the bubble formation, which may cause cell detachment and loss. We further evaluated cell integrity by examining the organization of the actin cytoskeleton. In both CO_2-treated and untreated cells, we observed a pattern of F-actin distribution within the cytoplasm that was consistent with cells spread onto a surface under static culture conditions (Figure 2c,d). Finally, we measured the incorporation of bromodeoxyuridine (BrdU) into hMSCs as an index of proliferative potential. The percentage of BrdU-labeled hMSCs after CO_2 treatment is around 70%, which is the same as hMSCs grown under static culture conditions (see Figure S1). These data, considered together, suggest that the CO_2 pressure has no obvious effects on the viability, attachment, or proliferation of hMSCs.

Next, we examined whether mouse ESCs (mESCs) can survive the same CO_2 treatment as the hMSCs. After CO_2 treatment, the mESCs were viable (Figure S2). All mESCs expressed Oct-4

[†] NSEC-CANPBD.
[‡] Department of Chemical and Biomolecular Engineering.
[§] Departments of Obstetrics & Gynecology and Biomedical Engineering.

10.1021/ja066157u CCC: $33.50

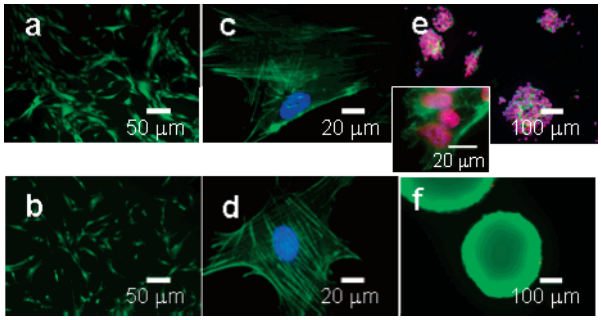

Figure 2. (a and b) Cell viability of hMSCs was shown using LIVE/DEAD staining (green, alive; red, dead); (c and d) cell attachment of hMSCs was shown by cytoskeleton F-actin expression (green; blue, DAPI stained nuclei); (e) the mESCs expressed Oct-4 after CO_2 treatment (pink; green, F-actin). The insert shows an enlarged view. (f) The mESCs with CO_2 treatment formed viable EBs after 7 days in medium without LIF: (a and c) without CO_2 treatment; (b, d, e, and f) with CO_2 treatment.

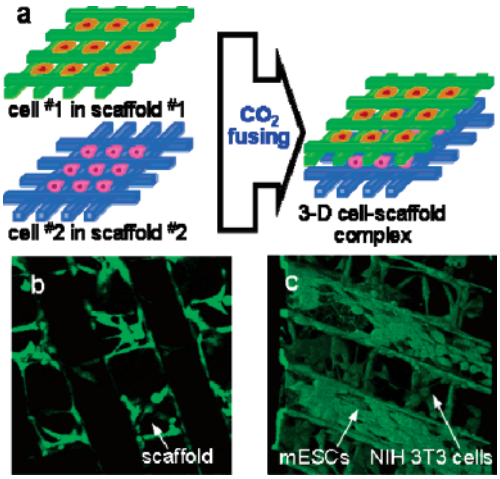

Figure 3. (a) Schematic CO_2-assisted assembly of 3-D cell-scaffold complex; confocal images of (b) the interface between bonded mESCs/NIH 3T3 cell-scaffold complex and (c) a 3-D view of the assembled cell-scaffold construct with high cellularity.

immunoreactivity in their nuclei (Figure 2e), the canonical marker of pluripotency. This indicated that all mESCs that survived the CO_2 treatment remained undifferentiated. After being cultured in a differentiating medium lacking a leukemia inhibitory factor (LIF), the mESCs that were exposed to CO_2 formed embryonic bodies (EBs) (Figure 2f), a necessary stage for differentiation, at a rate similar to the untreated cells. As predicted, the outgrowths of cells from EBs differentiated spontaneously into multiple lineages, as shown by the immunostaining of cells with lineage-specific antibodies. The expression of marker proteins of mesoderm (BMP-4), neuroectoderm (Pax-6), neural cells (nestin), cardiac muscle cells (Nkx2.5), and skeletal muscle cells (Myf5) demonstrated that the mESCs maintained their differentiation potential for early embryonic lineages, neurogenesis, cardiogenesis, and myogenesis after CO_2 treatment (Figure S3).

The finding that the CO_2 pressure has little effect on the behaviors of hMSCs and mESCs prompted us to further integrate multilayered PLGA scaffolds grown with different cell types into a 3-D complex using CO_2. As illustrated in Figure 3a, mESCs and NIH 3T3 fibroblasts were seeded on microfabricated PLGA scaffolds[3] and cultured, respectively. The scaffold with the mESCs and the scaffold with the NIH 3T3 fibroblasts were then stacked layer by layer and held with a compressive force of 55 kPa in culture medium. After being pressurized at 0.69 MPa for 15 min followed by the slow release of the CO_2 (see Supporting Information), the multiple scaffolds were assembled into a single 3-D construct, which was transferred to a fresh ES maintenance medium and cultured. This construct was stained by calcein AM to determine cell viability and cell distribution within the scaffolds (Figure 3b,c and movies in Supporting Information). Labeling with calcein AM in green revealed that the cells in the 3-D construct were viable after the CO_2-assisted assembly. The mESCs (round shapes) and the NIH 3T3 fibroblasts (stretched shapes) were controlled in the predesignated sites in the well-defined 3-D structure.

This approach is not restricted to hierarchical tissues. It can be used to manufacture heterogeneous but well-organized 3-D tissues as well. Multiple cell types can be cultured into cell-specific polymeric scaffolds with predefined structures, and these cell-scaffold building blocks can be coordinated into a large-scale system using a precise alignment and manipulation tool.[16] They then can be assembled into a complex construct using the CO_2-assisted assembly technique. Moreover, the protein and DNA we tested retained their integrity and functionality after CO_2 treatment (see Table S1). This suggests that signaling molecules can be integrated into the polymeric scaffold blocks selectively before cell seeding.[17] Therefore, the controlled and sustained delivery of the signaling molecules at the specific location can be realized in the complex, multifunctionalized tissue.

This CO_2-assisted assembly method offers an affordable and biologically permissive process, particularly for the simultaneous assembly of a large number of micro-/nanostructures containing temperature- and/or solvent-sensitive cells and biomolecules. In addition to tissue engineering, it can have wide applications in polymer-based biomedical micro-/nanodevices, such as in cell-based biochips, cell therapy, and drug and gene delivery devices.

Acknowledgment. This work was supported by the NSF-sponsored NSEC-CANPBD at OSU (Award No. EEC-0425626).

Supporting Information Available: Materials and methods, Figures S1−S3, Table S1, and two movies in avi format. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Desai, T. A.; Hansford, D. J.; Ferrari, M. *Biomol. Eng.* **2000**, *17*, 23−36.
(2) Lutolf, M. P.; Hubbell, J. A. *Nat. Biotechnol.* **2005**, *23*, 47−55.
(3) Yang, Y.; Basu, S.; Tomasko, D. L.; Lee, L. J.; Yang, S.-T. *Biomaterials* **2005**, *26*, 2585−2594.
(4) Liu, T. V.; Bhatia, S. N. *Adv. Drug Deliv. Rev.* **2004**, *56*, 1635−47.
(5) Tan, W.; Desai, T. A. *Biomaterials* **2003**, *25*, 1355−1364.
(6) Shea, L.; Smiley, E.; Bonadio, J.; Mooney, D. J. *Nat. Biotechnol.* **1999**, *17*, 551−554.
(7) King, K. R.; Wang, C.; Vacanti, J. P.; Borenstein, J. T. *Mater. Res. Soc. Symp. Proc.* **2002**, *729*, 3−8.
(8) Watson, M. S.; Whitaker, M. J.; Howdle, S. M.; Shakesheff, K. M. *Adv. Mater.* **2002**, *14*, 1802−1804.
(9) Tservistas, M.; Levy, M. S.; Lo-Yim, M. Y. A.; O'Kennedy, R. D.; York, P.; Humphrey, G. O.; Hoare, M. *Biotechnol. Bioeng.* **2001**, *72*, 12−18.
(10) Ginty, P. J.; Howard, D.; Rose, F. R. A. J.; Whitaker, M. J.; Barry, J. J. A.; Tighe, P.; Mutch, S. R.; Serhatkulu, G.; Oreffo, R. O. C.; Howdle, S. M.; Shakesheff, K. M. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 7426−7431.
(11) Abramowitz, H.; Shah, P. S.; Green, P. F.; Johnston, K. P. *Macromolecules* **2004**, *37*, 7316−7324.
(12) Cabanas, A.; Enciso, E.; Carbajo, M. C.; Torralvo, M. J.; Pando, C.; Renuncio, J. A. R. *Langmuir* **2006**, *22*, 8966−8974.
(13) Caskey, T.; Lesser, A. J.; McCarthy, T. J. *Polym. Eng. Sci.* **2001**, *41*, 2259−2265.
(14) Yang, Y.; Zeng, C.; Lee, L. J. *Adv. Mater.* **2004**, *16*, 560−564.
(15) Yang, Y.; Liu, D.; Xie, Y.; Lee, L. J.; Tomasko, D. L. *Adv. Mater.* in press.
(16) Hutmacher, D. W.; Sittinger, M.; Risbud, M. V. *Trends Biotechnol.* **2004**, *22*, 354−362.
(17) Richardson, T. P.; Peters, M. C.; Ennett, A. B.; Mooney, D. J. *Nat. Biotechnol.* **2001**, *19*, 1029−1034.

JA066157U