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Technical Note

Design of a Flow Perfusion Bioreactor System for Bone Tissue-Engineering Applications

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ABSTRACT

Several different bioreactors have been investigated for tissue-engineering applications. Among these bioreactors are the spinner flask and the rotating wall vessel reactor. In addition, a new type of culture system has been developed and investigated, the flow perfusion culture bioreactor. Flow perfusion culture offers several advantages, notably the ability to mitigate both external and internal diffusional limitations as well as to apply mechanical stress to the cultured cells. For such investigation, a flow perfusion culture system was designed and built. This design is the outgrowth of important design requirements and incorporates features crucial to successful experimentation with such a system.

OVERVIEW OF BIOREACTOR TYPES

A VARIETY OF bioreactor technologies have been documented for the culture of engineered tissues for *in vivo* implantation. All culture methods start from isolated cells seeded on three-dimensional scaffolds. The bioreactors aim to assist in the development of new tissue *in vitro* and to provide appropriate stimuli to the cultured cells. One of the simplest bioreactor designs is the spinner flask.[1–6] Scaffolds seeded with cells are attached to needles hanging from the cover of the flask. Sufficient medium is added to cover the scaffolds. Mixing of the medium is maintained with a magnetic stir bar at the bottom of the flask. The fluid dynamic environment within the stirred medium has been well characterized.[1,7] The convective forces generated by the magnetic stir bar mitigate the nutrient concentration gradients at the surface of the scaffolds. Cell/polymer constructs for cartilage tissue regeneration cultured for 5 weeks in spinner flasks were larger and contained more cells than did those grown in petri dishes.[1] Spinner flasks have also been successfully used for seeding cells on scaffolds with well-controlled cell densities.[2] Rat marrow stromal cells seeded on poly(DL-lactic-co-glycolic acid) porous scaffolds and cultured in spinner flasks demonstrated enhanced proliferation and expression of osteoblastic markers.[6]

Another bioreactor design that can be used with either microcarrier suspensions or scaffolds is the rotating wall vessel reactor originally designed to simulate microgravity effects.[8,9] The most popular type of this bioreactor is composed of two concentric cylinders whereas the microcarriers or the scaffolds are placed in the annular space between the two cylinders.[4] Gas exchange is allowed through the stationary inner cylinder while the outer cylinder is impermeable and rotates in a con-

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trollable fashion. Under carefully selected rotational rates the free falling of the scaffolds inside the bioreactor due to gravity can be balanced by the centrifugal forces due to the rotation of the outer cylinder, thus establishing microgravity-like culturing conditions. Chondrocytes seeded in scaffolds and cultured for 5 weeks using the rotating wall vessel bioreactor showed more glycosaminoglycan (GAG) production than those placed in a spinner flask.[10] Rat bone marrow stromal cells attached and formed three-dimensional aggregates in hollow microspheres in a rotating wall vessel bioreactor.[11] Osteoblast-like cells seeded on lighter-than-water polymer scaffolds and cultured in a rotating bioreactor showed increased alkaline phosphatase activity and mineralization.[12]

One bioreactor design that improves mass transfer at the interior of three-dimensional scaffolds is the flow perfusion bioreactor. This type of bioreactor uses a pump to perfuse medium continuously through the interconnected porous network of the seeded scaffold. The fluid path must be confined so as to ensure the flow path is through the scaffold, rather than around the edges. Bioreactors that employ the latter flow path, exchanging medium in the chamber around the scaffold, have been utilized before and have been termed "perfusion chambers."[4] This exchange of medium around the external surfaces of the scaffold, however, does not guarantee an exchange of medium within the porous confines of the scaffold interior.

The perfusion bioreactor offers enhanced transport of nutrients because it allows medium to be transported through the interconnected pores of the scaffolds. Murine K8 osteosarcoma cells seeded in three-dimensional collagen sponges and cultured in a flow perfusion bioreactor demonstrated enhanced viability, alkaline phosphatase activity, and mineralization.[13] Rat marrow stromal cells seeded on three-dimensional porous biodegradable polymer foams and cultured in a perfusion bioreactor resulted in better cell uniformity throughout the cultured foams and elevated alkaline phosphatase activity when compared with a spinner flask and a rotating wall vessel bioreactor.[5] A flow perfusion bioreactor was developed in our laboratory for three-dimensional osteoblast culture in porous scaffolds. Using this bioreactor calcified matrix production was dramatically increased in titanium fiber meshes seeded with marrow stromal osteoblasts over statically cultured constructs,[14,15] with the total calcium content of the cultured scaffolds increasing with increasing flow rate.[15]

FLOW PERFUSION BIOREACTOR

Motivation

The results of the studies in our laboratory indicated that currently available bioreactors are inadequate for fostering cell growth and mineralized matrix production throughout the seeded scaffolds.[6] The results also indicated that enhanced medium delivery and mechanical forces may potentially increase cell differentiation.[6] However, while bioreactors such as the spinner flask mitigated external diffusional limitations, internal diffusional limitations, that is, the inability of medium and other nutrients to penetrate within the porous network of the scaffold, remained. This resulted in cell growth and matrix production being confined to the external aspects of each scaffold.[6] For this reason, it was desirable to investigate flow perfusion culture as an alternative method that could potentially mitigate these limitations, especially for metabolically active cells such as osteoblasts.

In a flow perfusion bioreactor, medium is pumped through each scaffold continuously. In this manner, medium is delivered throughout each cultured scaffold (Fig. 1). A flow perfusion bioreactor offers several advantages for culturing scaffolds for tissue engineering. It provides enhanced delivery of nutrients throughout the entire scaffold by mitigating both external and internal diffusional limitations as fresh medium is not only delivered to each scaffold, but also throughout the internal structure of each scaffold. In addition, it offers a convenient way of providing mechanical stimulation to the cells by way of fluid shear stress (bone cells are known to be stimulated by mechanical signals).[16,17] The amount of shear stress experienced by cells cultured in a flow perfusion system can be varied simply by varying the flow rates through the system. Of course, depending on the porous structure, the local shear stresses experienced by individual cells will be variable and depend on the scaffold microarchitecture. A flow perfusion bioreactor thus extends the benefits offered by the spinner flask bioreactor. It gives external and internal mixing of the medium

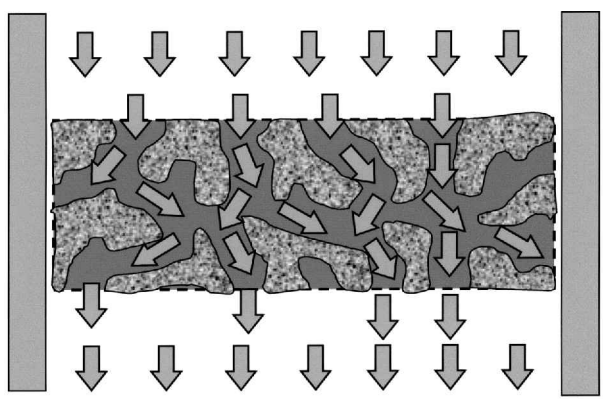

FIG. 1. Flow perfusion culture. In flow perfusion culture, the culture medium is forced through the internal porous network of the scaffold. This can mitigate internal diffusional limitations present in three-dimensional scaffolds to enhance nutrient delivery to and waste removal from the cultured cells.

for nutrient delivery to the cells and also exposes the cultured cells to mechanical forces. Thus, a flow perfusion bioreactor could conceivably allow marrow stromal osteoblasts to proliferate throughout a cultured scaffold through enhanced nutrient delivery/waste removal and also enhance osteoblastic differentiation through added mechanical stresses.[15] This would create a scaffold with a layer of cells and their associated matrix throughout.[15]

Design philosophy

To investigate the use of flow perfusion culture for bone tissue engineering, a flow perfusion culture system was developed in our laboratory. There are several requirements for a successful flow perfusion system design. First, it must deliver the flow through the scaffolds, minimizing the nonperfusing flow that goes around each cultured scaffold. Otherwise, it offers little advantage over the mixing provided by a reactor such as a spinner flask. Second, it must have a repeatable, controllable, and consistent rate of flow delivered to the constructs cultured. For comparisons both within and between experiments, consistency must be maintained to correctly draw conclusions about the results obtained. Third, it must be able to be sterilized and maintained in this sterile condition throughout the culture period. Again, contamination can alter the results, causing incorrect conclusions to be made. And, fourth, the system should be reasonable to operate. The more complicated and troublesome a system becomes, the more room for extraneous factors to unintentionally complicate the effects and thereby alter the results.

Design

Our flow perfusion bioreactor was machined from Plexiglas. Plexiglas is sterilizable by ethylene oxide gas exposure and offers the advantage of being relatively transparent, allowing the contents of each flow chamber to be visualized through the block. In addition, it is readily machinable to allow for the fabrication of the unit. The flow system body consists of individual flow chambers machined into one block (Fig. 2). The design of each chamber is shown in Fig. 3.

The scaffold is held in a cassette sandwiched between two neoprene O-rings. The O-rings are held tightly against the cassette by a screw top (Fig. 3). Medium enters through the top hole in the screw top, passes through the scaffold, and exits through the bottom. This top-to-bottom flow design is not accidental, but was created in such a manner to prevent bubbles from being trapped against the undersurface of the scaffold and altering the flow distribution. The complete flow system body consists of six of these chambers machined into one block of Plexiglas (Fig. 2).

The use of the cassette to hold the scaffold is important and a significant feature of the design. By simply machining new cassettes with different opening diameters it is possible to culture scaffolds of different sizes with relative ease. In addition, the casting, leaching, sterilizing, prewetting, coating, and seeding of the scaffolds can occur with the scaffolds within the cassettes, ensuring a tight fit between scaffold and cassette, which prevents undesirable nonperfusing flow. These concepts are illustrated in Fig. 4.

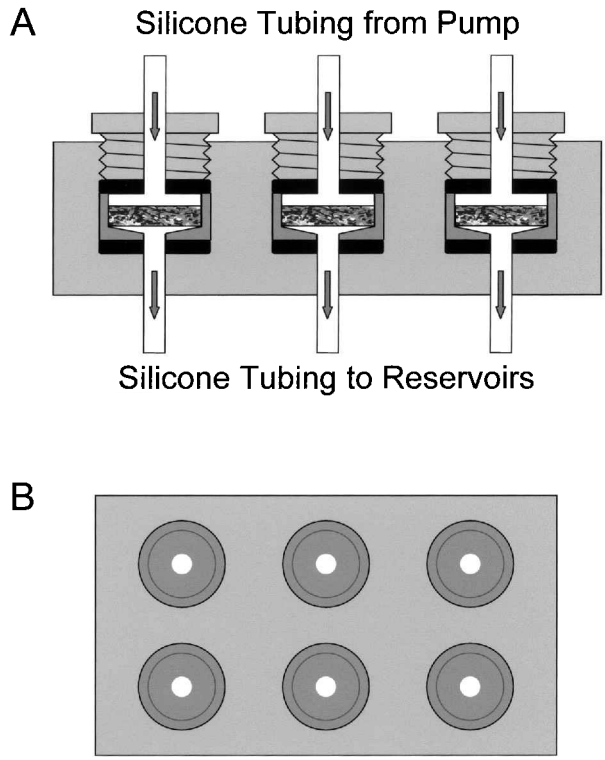

FIG. 2. Flow system block diagram. (**A**) Arrangement of flow chambers in flow system block (side view). (**B**) Arrangement of flow chambers in flow system block (overhead view).

The flow system circuit consists of six flow circuits running in parallel off of one multichannel pump (Cole Parmer, Vernon Hills, IL) drawing from a common reservoir. Medium is drawn from the first reservoir, pumped through each flow chamber, and then returned to the second medium reservoir. From here the medium flows, driven by gravity head, back to the first reservoir. This flow system circuit is illustrated in Fig. 5. Our design utilized six chambers in the flow chamber block because the pumps used in our laboratory have six pump heads. Certainly, using multichannel pumps with more than six pump heads can allow for the addition of more flow chambers.

The tubing used to connect each component in the circuit is platinum-cured silicone tubing (Masterflex tubing; Cole Parmer). The platinum curing minimizes the amount

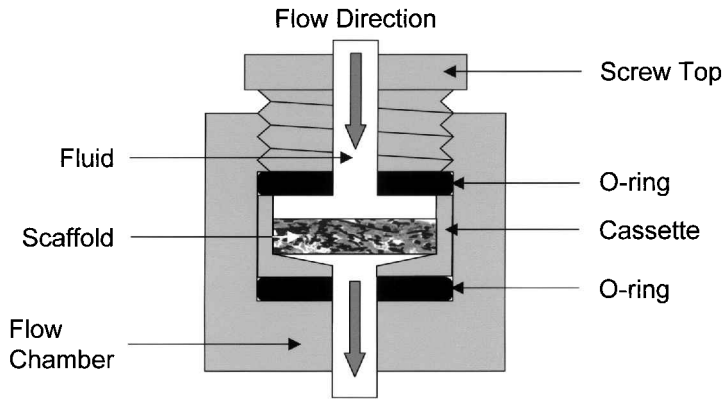

FIG. 3. Flow chamber and cassette diagram. Illustrated here is the design of the flow chamber in the flow perfusion culture system. Six of these chambers are molded into each block, allowing for the culture of six specimens at a time with each flow system. The scaffold is press-fit into a custom-machined Plexiglas cassette. These cassettes are machined specifically to the diameter of the scaffolds used and can be made with different diameters for investigating scaffolds of varying dimensions. The cassette with scaffold is sealed in place by two neoprene O-rings above and below the cassette. This three-part assembly (cassette and two O-rings) is then held in place by a Plexiglas screwtop. Silicone tubing then connects each of these flow chambers to the pump and reservoir systems.

of leachable chemicals, an important consideration in tissue culture work. In addition, this silicone tubing is relatively low-protein binding. Most importantly, the tubing is highly gas permeable to both carbon dioxide and oxygen, permitting proper gas exchange for oxygen delivery and maintenance of proper culture medium pH.

The pump driving the flow is a six-channel peristaltic pump (Cole Parmer). This pump gives accurate and consistent flow rates from 0.1 to 10 mL/min with the tubing size used in our system (Cole Parmer L/S 16). Because of the peristaltic nature of the pump and the relatively lower mechanical durability of silicone tubing, neoprene tubing (Pharmed tubing; Cole Parmer), a more rigid but non-gas-permeable tubing, is used for a short segment of the circuit within the pump.

The medium reservoir consists of two side-armed flasks (Bellco Glass, Vineland, NJ) modified by the addition of a short glass stem at the bottom edge to allow for their connection by a short section of silicone tubing. Medium returning from each flow chamber flows through a needle piercing the top of the first flask. The droplet then falls to the medium level below. This design allows for the easy and quick visual verification of flow and also enhances the gas exchange of the medium. The medium then flows under gravity to the second flask, where it is drawn up through the glass pipettes and pumped again through the system. This reservoir arrangement is shown in Fig. 6.

This two-flask design also facilitates changing of the medium within the system. It is possible to achieve an essentially complete change of the system medium by simply closing the connection between the two reservoirs, emptying both reservoirs, filling the first reservoir with fresh medium, and pumping the old medium into the sec-

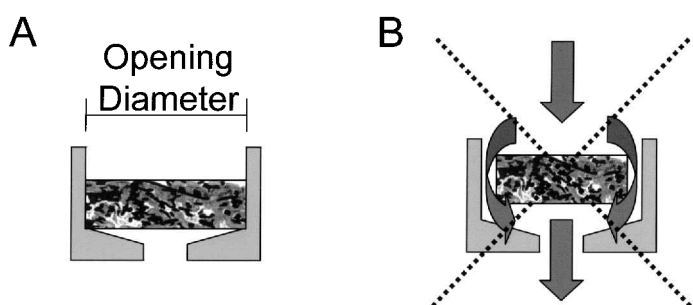

FIG. 4. Illustration of cassette fundamentals. (**A**) It is of special importance that the opening diameter of the cassette be made with a tight tolerance to the dimensions of the scaffold being cultured. (**B**) Failure to ensure a tight seal between the edges of the scaffold and the cassette will allow the perfused medium to follow the path of least resistance, which is usually around the scaffold. This is referred to as "nonperfusing flow."

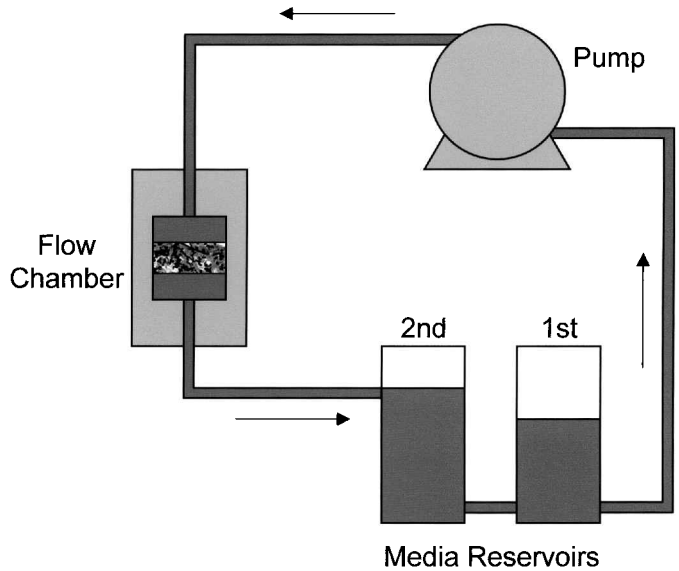

FIG. 5. Flow perfusion system flow circuit diagram. Medium is drawn from the first medium reservoir by the actions of the peristaltic roller pump. The medium is then pumped downward through the flow chamber. On exiting the flow chamber it flows to the second medium reservoir. Under the force of gravity, it then returns to the first medium reservoir, completing the circuit. It should be noted that the actual flow system uses six independent pumping circuits drawing from a common reservoir system pumped by a six-channel pump. For the sake of simplicity, only one circuit is illustrated here. This diagram also is useful for illustrating the manner in which the medium is changed. The procedure is as follows: the connection between the two reservoirs is closed. The first reservoir is drained of its old medium, rinsed, and then refilled with fresh medium. The pump is then run for a sufficient length of time to flush the old medium out of the system. All the medium is then drained from the second reservoir. At this point, the connection between the two reservoirs is established and the essentially complete medium change is finished.

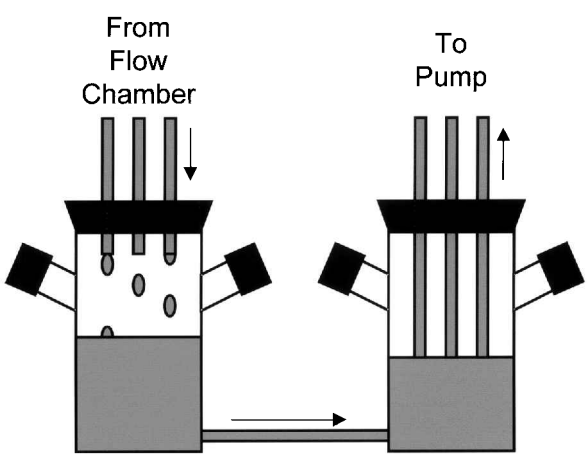

FIG. 6. Diagram of reservoir arrangement. Medium returns from the flow chambers and returns to the left reservoir by falling as droplets to the liquid surface. The air in the reservoir through which the droplets fall is open to the incubator atmosphere via filter caps on the ends of the flask arms. Under the gravity head, the medium flows to the right reservoir, where it is drawn up out of the reservoir by the pump and perfused back through the flow chambers.

ond reservoir, where it is removed before reestablishing the connection between the two reservoirs. The medium is easily added or removed via the side arms of each flask, which are covered with removable gas-permeable filter caps when in use.

The entire system is readily sterilizable. The flow system body with the cassettes, all made of Plexiglas, must be sterilized by ethylene oxide. The tubing, reservoir flasks, and all other parts are sterilizable by autoclaving. Because of this, the majority of the flow system can be assembled and sterilized as one piece before placing the seeded scaffolds into the system. This aspect of the design enhances usability and also limits handling of components during start-up, thereby lessening opportunities for the introduction of contaminants.

CONCLUSIONS

A flow perfusion system has been designed and built. Through the use of its cassette system for holding the individual scaffolds it ensures perfusion flow through each scaffold. By using a multichannel pump with each flow

chamber isolated on its own pumping circuit it is possible to repeatedly deliver accurate, controlled flow rates. All components of the flow system are sterilizable and set-up has been optimized to minimize the introduction of contaminants. The dual-reservoir system allows for easy access for medium removal and addition and complete purging of the medium at changes. These aspects are important for limiting contamination. In addition, the system has been simplified to include a minimum number of parts, allowing for ease of handling. This flow system can thus be used for experimentation and valid conclusions can be made from the results obtained. Undoubtedly, as with any experimental set-up, further opportunities for optimization will become known and incorporated into future designs.

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REFERENCES

1. Vunjak-Novakovic, G., Freed, L.E., Biron, R.J., and Langer, R. Effects of mixing on the composition and morphology of tissue-engineered cartilage. AIChE J. **42,** 850, 1996.
2. Vunjak-Novakovic, G., Obradovic, B., Martin, I., Bursac, P.M., Langer, R., and Freed, L.E. Dynamic cell seeding of polymer scaffolds for cartilage tissue engineering. Biotechnol. Prog. **14,** 193, 1998.
3. Vunjak-Novakovic, G., Martin, I., Obradovic, B., Treppo, S., Grodzinsky, A.J., Langer, R., and Freed, L.E. Bioreactor cultivation conditions modulate the composition and the mechanical properties of tissue engineered cartilage. J. Orthop. Res. **17,** 130, 1999.
4. Freed, L.E., and Vunjak-Novakovic, G. Tissue engineering bioreactors. In: Lanza, R.P., Langer, R., and Vacanti, J., eds. Principles of Tissue Engineering. San Diego, CA: Academic Press, 2000, pp. 143–156.
5. Goldstein, A.S., Juarez, T.M., Helmke, C.D., Gustin, M.C., and Mikos, A.G. Effect of convection on osteoblastic cell growth and function and biodegradable polymer foam scaffolds. Biomaterials **11,** 1279, 2001.
6. Sikavitsas, V.I., Bancroft, G.N., and Mikos, A.G. Formation of three-dimensional cell/polymer constructs for bone tissue engineering in a spinner flask and a rotating wall vessel bioreactor. J. Biomed. Mater. Res. **62,** 136, 2002.
7. Cherry, R.S., and Papoutsakis, T. Physical mechanisms of cell damage in microcarrier cell culture bioreactors. Biotech. Bioeng. **32,** 1001, 1988.
8. Schwarz, R.P., Goodwin, T.J., and Wolf, D.A. Cell culture for three-dimensional modeling in rotating-wall vessels: An application of simulated microgravity. J. Tissue Cult. Methods **14,** 51, 1992.
9. Freed, L.E., and Vunjak-Novakovic G. Cultivation of cell–polymer constructs in simulated microgravity. Biotech. Bioeng. **46,** 306, 1995.
10. Freed, L.E., and Vunjak-Novakovic, G. Microgravity tissue engineering. In Vitro Cell Dev. Biol. Anim. **33,** 381, 1997.
11. Qiu, Q.Q., Ducheyne, P., and Ayyaswamy, P.S. Fabrication, characterization and evaluation of bioceramic hollow microspheres used as microcarriers for 3-D bone tissue formation in rotating bioreactors. Biomaterials **20,** 989, 1999.
12. Botchwey, E.A., Pollack, S.R., Levine, E.M., and Laurencin, C.T. Bone tissue engineering in a rotating bioreactor using a microcarrier matrix system. J. Biomed. Mater. Res. **55,** 242, 2001.
13. Mueller, S.M., Mizuno, S., Gerstenfeld, L.C., and Glowacki, J. Medium perfusion enhances osteogenesis by murine osteosarcoma cells in three-dimensional collagen sponges. J. Bone Miner. Res. **12,** 2118, 1999.
14. Van Den Dolder, J., Bancroft, G.N., Sikavitsas, V.I., Spauwen, P.H.M., Jansen, J.A., and Mikos, A.G. Flow perfusion culture of marrow stromal osteoblasts in titanium fiber mesh. J. Biomed. Mater. Res. **64A**, 235, 2003.
15. Bancroft, G.N., Sikavitsas, V.I., Van Den Dolder, J., Sheffield, T.L., Ambrose, C.G., Jansen, J.A., and Mikos, A.G. Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. Proc. Natl. Acad. Sci. U.S.A. **99,** 12600, 2002.
16. Hillsley, M.V., and Frangos, J.A. Bone tissue engineering: The role of interstitial fluid flow. Biotech. Bioeng. **43,** 573, 1994.
17. Sikavitsas, V.I., Temenoff, J.S., and Mikos, A.G. Biomaterials and bone mechanotransduction. Biomaterials **22,** 2581, 2001.

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