Effective cell collection method using collagenase and ultrasonic vibration

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This study proposes a novel cell collection method based on collagenase treatment and ultrasonic vibration. The method collects calf chondrocytes from a reusable metal cell culture substrate. To develop our concept, we calculated the natural vibration modes of the cell culture substrate by a finite element method, and conducted eigenvalue and piezoelectric-structural analyses. Selecting the first out-of-plane vibration mode of the substrate, which has a single nodal circle, we designed and fabricated the cell collection device. The excited vibration mode properly realized our intentions. We then evaluated the cell collection ratio and the growth response, and observed the morphology of the collected cells. The collagenase and ultrasonic vibration treatment collected comparable numbers of cells to conventional trypsin and pipetting treatment, but improved the proliferating cell statistics. Morphological observations revealed that the membranes of cells collected by the proposed method remain intact; consequently, the cells are larger and rougher than cells collected by the conventional method. Therefore, we present a promising cell collection method for adhesive cell culturing process. © 2014 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4899054>]

I. INTRODUCTION

Cell culturing is an important aspect of tissue engineering, which promises to generate hybrid artificial organs and autotransplants. However, if cultured cells are to be widely used in clinics, their yield ratio and activity must be boosted by improved cell culturing techniques.^{1,2} The number and activity of the cultured cells chiefly decide the success of a cell culturing process and are affected by several chemical/physical factors, such as substrate quality,^{3–5} additional growth factors,^{6,7} and mechanical stimuli.^{8–10} Most existing cell culture methods are designed to improve the number and activity of cells during the culturing process. Alternatively, these factors may be increased by improving the cell collection method.

For example, cells adhered to substrates, such as chondrocytes, fibroblasts, and osteoblasts, must be detached from the culture substrates after the culturing process. Cell detachment is usually performed by enzymatic treatment followed by physical collection such as pipetting. However, cell membranes are easily damaged by standard trypsinization (detachment by trypsin, a protein hydrolyzing enzyme) followed by pipetting.¹¹ Hirai *et al.* reported that prolonged trypsin treatment damages cell membranes by partially hydrolyzing various membrane-expressed proteins, impairing cellular activity, and reducing cell proliferation.^{12,13}

To remove cells adhered to substrates, a novel procedure for detaching and collecting cells from the culture substrate is therefore required. One new approach is based on temperature-responsive polymer,^{14,15} which reversibly alters its hydrophobic/hydrophilic nature in response to cultivation temperature. The seeded cells adhere to the substrate and proliferate at 37 °C. If the temperature is lowered, the surface is rapidly hydrated, spontaneously releasing the cultured cells without requiring proteolysis enzyme treatment. Another approach is to culture the cells

on a Fe-alginate substrate.¹⁶ Under physiological conditions, the Fe-alginate scaffold is easily dissolved by chelating agents such as citrate, which facilitate exchange of cross-linked ions. In this approach, cells are released by disintegration of the alginate gel. The major disadvantage of these collection methods is limited substrate availability; that is, temperature-responsive polymers and the gels in Fe-alginate substrates cannot be reused after surface modification. Consequently, these collection methods are not suitable for various types of cell cultures or repetitive use. They are unsuitable for sustainable clinical use.

To resolve the reusability problem, this study proposes a novel cell collection method using collagenase and ultrasonic vibration. First, we design and fabricate a cell culturing device made of biocompatible stainless steel, whose culturing surface can vibrate at high frequency. Calf chondrocytes are cultured in this device, then released by a novel combination of collagenase and ultrasonic vibration. To confirm the effectiveness of the proposed method, we investigate the cell proliferation and collection rate in a series of experiments.

II. CELL COLLECTION DEVICE USING ULTRASONIC VIBRATION

A. Concept

As mentioned in the Introduction, collecting adherent cells from a cultivation base is an important procedure in tissue engineering. We propose that removing cells from the cultivation base by ultrasonic vibration will reduce cell damage, which is problematic in conventional cell collection. This idea is conceptualized in Fig. 1. In tissue engineering, ultrasonic vibration is frequently used to break up cells;^{17,18} here, we suggest that adequate vibration stimuli may effectively detach cells from the base.

B. Design

To realize an effective cell collection device using ultrasonic vibration, we apply vibrations at the natural frequency of the cultivation substrate. Therefore, we first design the vibration mode of the cultivation substrate. The constraint conditions of the cell cultivation device are

FIG. 1. Image of cell detachment by using ultrasonic vibration. (a) Cells are adhered to substrate. (b) Substrate is vibrated with high frequency. (c) Cells are detached from substrate.

FIG. 2. Cell cultivation device has a metal cultivation plate, a silicone rubber wall, a top plate, and a silicone rubber cover.

the metal cultivation plate, silicone rubber wall, top plate, and silicone rubber cover, assembled as shown in Fig. 2. Although metal cultivation surfaces are rarely used in tissue engineering researches, we previously proved that the metal surface modified with Fine Particle Peening (FPP) treatment is comparable to conventional plastic cell cultivation surface^{19,20} and is therefore suitable for non-disposable cultivation equipment.

To realize our concept, we calculated the natural vibration modes of the metal cultivation plate using the finite element method software ANSYS 15.0. The dimensions of the metal plate are shown in Fig. 3. The edges of the bolt holes at each corner are fixed in the finite element model. The Young's modulus, Poisson's ratio, and density of the metal plate are $19.3 \times 10^{10} \text{ N/m}^2$, 0.3, and $7.98 \times 10^3 \text{ kg/m}^3$, respectively (where the density is that of AISI 316L austenitic stainless steel used in our previous study).¹⁷ From the eigenvalue analysis, we adopt the first out-of-plane vibration mode, which has a single nodal circle and resonates at 17.3 kHz (see Fig. 4).

To effectively excite the adopted vibration mode, we designed a piezoelectric ceramic disk (outer diameter = 20 mm; thickness = 0.5 mm) to be glued onto the metal plate. The alignment

FIG. 3. Dimension of the metal cultivation plate: (a) plan view and (b) elevational view. All dimensions are in mm.

FIG. 4. First out-of-plane vibration mode obtained by eigenvalue analysis using ANSYS. The color bar indicates the normalized displacement in the out-of-plane direction by the maximum value.

and dimensions of the piezoelectric ceramic disk glued onto the metal plate are presented in Fig. 5. Note that we also model a 0.05 mm-thick epoxy adhesion layer between the plate and the disk. The Young's modulus, Poisson's ratio, and density of the piezoelectric ceramic disk are $3.56 \times 10^{-9} \text{ N/m}^2$, 0.29, and $7.6 \times 10^3 \text{ kg/m}^3$, respectively, and those of the adhesion layer are $2.5 \times 10^9 \text{ N/m}^2$, 0.4, and $2.3 \times 10^3 \text{ kg/m}^3$, respectively. The adequacy of this design was confirmed by piezoelectric-structural analysis using ANSYS. The piezoelectric ceramic disk was supplied with $10 \text{ V}_{\text{p-p}}$ AC at 19.1 kHz. As shown in Fig. 6, the vibration mode excited by the 19.1 kHz AC input matches the first out-of-plane vibration mode obtained by eigenvalue analysis (Fig. 4). The input and natural mode frequencies differ mainly because the piezoelectric ceramic disk adds thickness to the model, thereby increasing its resonant frequency.

FIG. 5. Dimension of the cell culture plate with piezoelectric ceramic disk. The metal plate and the piezoelectric ceramic disk are bonded by epoxy adhesive, which have a thickness of 0.05 mm. (a) Plan view and (b) elevational view. All dimensions are in mm.

FIG. 6. Result of the piezoelectric-structural analysis using ANSYS. The vibration mode shape excited with 19.1 kHz AC input. The color bar indicates the vibration displacement in the out-of-plane.

C. Fabrication

Based on the finite element model, a cell collection device using ultrasonic vibration was developed. Figs. 7(a) and 7(b) are a schematic of the cell collection device comprising a silicone rubber cover, a top cover, a silicone rubber wall, a metal cell culture substrate, and a piezoelectric ceramic disk. The copper tape inputs the AC voltage input, while the substrate is connected to ground. The silicone rubber wall and substrate collectively configure the cell culturing chamber ($\phi 40$ mm). The depth of the cell culturing chamber is 10 mm.

FIG. 7. Schematic illustration of ultrasonic vibration cell collection device. (a) Structure of the device, (b) back side of the device, and (c) photograph of fabricated cell collection device.

TABLE I. Conditions for FPP treatment.

Peening pressure (MPa)	0.6
Peening time (s)	30
Nozzle distance (mm)	100
Shot particle	Al ₂ O ₃
Particle supply rate (g/s)	1

The cell culture substrate is constructed from AISI 316L austenitic stainless steel, a commonly used biomaterial. The FPP treatment controls the surface microstructure of the substrate,²⁰ ensuring a smooth culturing surface. The conditions of the FPP treatment are summarized in Table I. The shot particles are aluminum (III) oxide (alumina) particles of diameter 20 μm . The fabricated cell collection device is shown in Fig. 7(c).

D. Vibration characteristics

The vibration characteristics of the substrate were evaluated in experiments. Fig. 8 shows the experimental setup. The first out-of-plane vibration mode of the substrate was excited by applying the AC input to the piezoelectric ceramic disk, and vibrations were measured by a laser Doppler vibrometer (CLV-3D, PI Polytech). Fig. 9 shows the relationship between the input frequency f and the vibration amplitude A at the center of the substrate, for an input voltage of 10 V_{p-p}. The amplitude A is calculated from the measured velocity amplitude A_v and the angular frequency ω ($=2\pi f$),

$$A = \frac{A_v}{\omega}. \quad (1)$$

At 10 V_{p-p} operating voltage, the resonance frequency was identified as 17.2 kHz. The shape of the vibration mode excited at 17.2 kHz AC input is shown in Fig. 10. Since the horizontal axis represents the distance from the center of the substrate, the excited vibration mode clearly corresponds to the first out-of-plane vibration mode with a single nodal circle (located approximately 11 mm from the center).

FIG. 8. Schematic illustration of experimental setup for characterizing ultrasonic vibration. The vibration of the cell culturing device is measured by the LDV.

FIG. 9. Relationship between the vibration amplitude and the driving frequency of the ultrasonic vibration cell collection device with driving voltage of 10Vp-p.

The measured resonance frequency is 9.94% lower than that obtained by piezoelectric-structural analysis. This discrepancy may be explained by three reasons. First, the mode mass of the device may be increased by contact between the metal cell culture substrate and the silicone rubber wall. Second, the resonance frequency is lowered by the bubbles in adhesion layer that appeared during the bonding process of the substrate and piezoelectric ceramic disk. The bubbles make stiffness of the adhesion layer lower. Third, because the actual substrate is fixed by bolts and nuts, the edges of the holes may become more flexible than admitted by the model boundary conditions.

Despite these differences between the model and the fabricated device, the excited vibration mode properly reproduces our intended vibration pattern.

III. EXPERIMENTAL PROCEDURE

A. Preparation of cells

The target cells, calf chondrocytes, were harvested from the knee joints of 4–6 week-old calves obtained from a local abattoir. The articular cartilage was diced into $\sim 1 \text{ mm}^3$ pieces and gently shaken in Dulbecco's modified Eagle's medium/Ham's F-12 supplemented with 10% fetal bovine serum (FBS), 0.15% collagenase type I, and Antibiotic-Antimycotic for 18 h at 37 °C. Cells were then isolated from the tissue by centrifugation,⁹ and suspended and cultured in feed medium (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (D-MEM/F12) supplemented with 10% FBS) in a 5% CO₂ humidified atmosphere incubator at 37 °C. Cell passage was performed at 3-day intervals by trypsinization in 0.05% trypsin and 0.02% EDTA in Ca-Mg-free saline with pipetting.

The completely dedifferentiated third-passage chondrocytes²¹ were seeded in the cell collection device described in Sec. II. The seeded culture (1.5×10^5 cells in 500 μl medium) is incubated for 24 h in a 5% CO₂ humidified atmosphere incubator at 37 °C. The incubated sample is the initial sample used in the cell collecting experiments.

B. Cell collecting methods

To prove that our proposed method collects a high proportion of the cells and preserves cellular activity, we compared the results of six cell collecting experiments. Here, the substrate was excited by ultrasonic vibrations at 17.2 kHz. The input voltage was adjusted to ensure a peak vibrational amplitude (at the center of the substrate) of 1 μm . The cell collection

(a) Result of the piezoelectric-structural analysis with 19.1 kHz AC input.

(b) Measured vibration distribution of the substrate with 17.2 kHz AC input (mean $\pm$ SD, $n = 4$)

FIG. 10. Comparison of (a) results of the piezoelectric-structural analysis with (b) measured vibration distribution of the device.

treatments were carried out in a 5% CO₂ humidified atmosphere incubator at 37 °C. The cell culture chamber was that used in the ultrasonic vibration treatment.

1. Collagenase–ultrasonic vibration (USV) treatment (proposed method)

Chondrocytes cultured on the substrate were collected by 0.10% collagenase treatment with ultrasonic vibration for 3 min.

2. Trypsin–pipetting treatment (conventional method)

Cells were collected by traditional trypsin-EDTA treatment and pipetting. Specifically, chondrocytes were exposed to 0.05% trypsin-EDTA for 3 min and then collected by pipetting.

3. Trypsin–USV treatment

Chondrocytes were collected by 0.05% trypsin-EDTA treatment with ultrasonic vibration for 3 min.

4. Collagenase-pipetting treatment

Chondrocytes were exposed to 0.10% collagenase for 3 min and then collected by pipetting.

5. Collagenase treatment

Chondrocytes were collected solely by 3 min exposure to 0.10% collagenase.

6. USV treatment

Chondrocytes were collected solely by 3 min application of ultrasonic vibration.

IV. RESULTS AND DISCUSSION

First, we demonstrate the efficacy of the proposed method by comparing the collagenase–USV treatment and trypsin-pipetting treatments. This discussion is followed by a detailed morphological evaluation of the treatments.

A. Comparison of proposed and conventional cell collecting methods

To compare the cell collection performances of the collagenase–USV and trypsin-pipetting treatments, we first counted the number of cells recovered by each treatment. Recovered cells were counted in a hemocytometer, and the results are shown in Fig. 11. Both treatments retrieved similar numbers of cells, indicating that the collagenase–USV treatment offers no superior advantage over the conventional method in terms of cell recovery.

We supplemented the above comparison with fluorescence microscopy observation of the cells remaining on the substrate after the collecting process. Fluorescence microscopy images of vinculin-stained cells remaining on the substrate are shown in Fig. 12 (for reference, non-collected cells grown on the substrate are shown in Fig. 12(c)). No vinculin is observed on the substrate after collagenase–USV treatment, consistent with the selective breakdown of collagen (a substrate protein secreted by chondrocytes) by collagenase. The small vinculin fluorescence after trypsin-pipetting treatment probably arises because the lamellipodia are broken down by trypsin before breakdown of the extracellular matrix. This suggests that the cells are much more damaged by the conventional trypsin-pipetting treatment than by the proposed

FIG. 11. Comparison of number of collected cells. Chondrocytes were seeded into the ultrasonic vibration cell culturing device and cultured for 24 h. Cells were then collected by either collagenase-USV treatment or trypsin-pipetting treatment (mean $\pm$ SD, $n = 8$).

FIG. 12. Vinculin fluorescent microscope images of chondrocytes on substrate after collecting cells by each method. (a) Trypsin-pipetting treatment. (b) Collagenase-USV treatment. (c) Reference: before collecting process.

collagenase-USV treatment. Because ultrasonic vibration generates heat, and chondrocytes are killed at temperatures exceeding 42°C , the temperature change was measured with a thermistor. Fig. 13 shows the temperature history during 3 min application of ultrasonic vibration. Since the temperature is constant, we conclude that the cells are unaffected by temperature changes.

To confirm this hypothesis, we evaluated the cell activity of the collected cells. The chondrocytes were completely dedifferentiated; that is, they were specialized to proliferate,²² and thus ideally suited for evaluating cell activity. Chondrocytes (1.5×10^4 cells) collected by the

FIG. 13. Relationship between the substrate temperature and vibration time of the ultrasonic vibration cell retrieval device.

FIG. 14. Comparison of the number of cells collected. 1.5×10^4 cells collected by each method were reseeded and cultured for 72 h (mean $\pm$ SD, $n = 8$, **: $p < 0.01$).

collagenase-USV and trypsin-pipetting treatments were independently reseeded in 24-well plates, and counted 72 h later. As shown in Fig. 14, the collagenase-USV treatment yielded statistically more cells at 72 h than standard trypsin-pipetting treatment. This result indicates higher activity of cells collected by the proposed method than by the conventional method.

FIG. 15. (a) and (b) Modulation contrast microscope images of chondrocyte. (c) Comparison of the area of cells collected (mean $\pm$ SD, $n = 15$, **: $p < 0.01$).

The above experimental results highlight the potential applicability of the proposed collagenase-USV treatment as a cell collecting method.

B. Morphology observation of collected cells

If cells are damaged during the collecting process, their activity reduces after reseeding. The efficacy of cell collecting methods must be confirmed in cell morphology examinations. Modulation contrast microscope images of cells collected by each method, and their measured areas, are shown in Fig. 15. Cells collected by the collagenase-USV treatment are statistically larger than cells collected by the trypsin-pipetting treatment, suggesting that trypsinization targets the cell membrane. To evaluate the cell states in detail, the cell surfaces were prepared by t-butyl alcohol freeze-drying method and observed under a scanning electron microscope (SEM).²³ SEM images of cells taken 3 min after reseeding on a dish are shown in Fig. 16. The cells collected by the collagenase-USV treatment show rough outer surfaces with numerous microvilli (Fig. 16(a)). By contrast, the cells collected by the trypsin-pipetting treatment show smooth outer surfaces (Fig. 16(b)), again possibly indicating the breakdown of cell membranes by trypsinization. Damage to cell membrane by trypsin reduces cell proliferation.²⁴

In addition to the cell morphology, we observed the grown filopodia of cells 2 h after reseeding (Fig. 17). The state of the filopodia may reflect the proliferative ability of cells.²⁵ The filopodia of cells collected by the collagenase-USV treatment were denser than those of cells collected by the trypsin-pipetting treatment.

The cell collecting sequences are illustrated in Fig. 18. Collagenase breaks the peptide bonds of collagen (contained within the extracellular matrix), leaving the cell membrane intact. Consequently, cells collected after collagenase digestion are bigger and rougher than those exposed to trypsin, which hydrolyses cell membrane proteins.

FIG. 16. SEM images of chondrocyte on dish. (a) Collagenase and ultrasonic vibration treatment and (b) trypsin and pipetting treatment.

(a) Collagenase-USV treatment.

(b) Trypsin-pipetting treatment.

FIG. 17. SEM images of chondrocyte on dish after 2 h. (a) Collagenase and ultrasonic vibration treatment and (b) trypsin and pipetting treatment.

C. Effects of collagenase and ultrasonic vibration on cell collecting rate and proliferation

Although collagenase-USV treatment emerges as a promising cell collecting method in this study, an important question remains. Which of collagenase or ultrasonic vibration is chiefly responsible for cell retrieval? Or must collagenase and ultrasonic vibration be combined for successful cell retrieval?

FIG. 18. Schematic illustration of cell collecting sequence by collagenase and ultrasonic vibration treatment (a)–(c) and by trypsin and pipetting treatment (d)–(f). (a) Collagenase break down extra cellular matrix, (b) cell is detached from substrate by vibration, and (c) cell membrane is not damaged. (d) Trypsin break down extra cellular matrix and cell membrane, (e) cell is detached from substrate by water pressure, and (f) cell membrane is damaged.

To answer this question, we first compared the collagenase-USV, trypsin-USV, and USV treatments mentioned in Sec. III B. The number of cells retrieved by each treatment is shown in Fig. 19(a). The collagenase and trypsin-USV treatments retrieved statistically more cells than USV treatment alone. We then tested the activity (proliferation) of the collected cells as described in Sec. IV A. The numbers of cells at 72 h after reseeding are shown in Fig. 19(b). According to this figure, collagenase-USV treatment yields statistically higher numbers of cells than the other treatments, indicating that this treatment maximizes cell proliferation. USV treatment alone is relatively ineffective. Reportedly, cells collected a short time later have weak adhesive power; that is, deteriorated growth potential.²⁶ Only such weakly adhered cells could be collected by the USV treatment.

The results of Fig. 19 suggest that the collagenase-USV treatment, but not ultrasonic vibration alone, is effective for cell collection.

To determine whether ultrasonic vibration is required for effective cell collection, we collected cells by the collagenase-USV, collagenase-pipetting, and collagenase treatments

(a) Comparison of number of collected cells by using USV.

(b) Comparison of number of proliferated cells by using USV.

FIG. 19. (a) Comparison of number of collected cells. Cells are seeded into cell culturing device and cultured for 24 h, and then collected by either collagenase-USV treatment, trypsin-USV treatment, or USV treatment. (b) Comparison of the number of cells. Chondrocytes are re-seeded after collected by either collagenase-ultrasonic vibration treatment, trypsin-ultrasonic vibration treatment, or ultrasonic vibration treatment, and cultured for 72 h (mean $\pm$ SD, $n = 4$, **: $p < 0.01$).

mentioned in Sec. III B. The numbers of cells retrieved by each treatment are shown in Fig. 20(a). The collagenase-USV treatment retrieves statistically higher numbers of cells than the other treatments. Clearly, supplementing collagenase treatment with ultrasonic vibration raises the efficiency of cell collection.

We also tested the activity of the collected cells, as described in Sec. IV A. The numbers of cells at 72 h after reseeding in a 24-well plate are shown in Fig. 20(b). Although cells retrieved by collagenase-USV and collagenase-pipetting treatments similarly proliferate, cells retrieved by collagenase treatment proliferate less effectively. Given that collagenase selectively breaks the peptide bonds of collagen, this result indicates that collagenase treatment is too mild to detach the cells from the substrate.

From the above results and discussion, we conclude that collagenase treatment must be combined with ultrasonic vibration for effective cell collecting. The collagenase-USV treatment improves both the number and activity of the collected cells.

FIG. 20. (a) Comparison of the number of cells. Chondrocytes are reseeded after detachment by either collagenase-USV, collagenase-pipetting treatment, or collagenase treatment and cultured for 72 h. (b) Comparison of the number of cells. Chondrocytes are reseeded after detachment by either collagenase-USV, collagenase pipetting treatment, or collagenase treatment and cultured for 72 h (mean $\pm$ SD, $n = 4$, **: $p < 0.01$).

V. CONCLUSION

In this paper, we proposed a novel method using collagenase treatment and ultrasonic vibration for collecting calf chondrocytes from a solid surface. The efficacy of the method was experimentally verified. However, although the proposed collagenase–USV treatment is a promising cell collecting method, the device should be improved for clinical use. Since we used the out-of-plane flexural vibration mode with a single nodal circle, the vibration amplitude is non-uniform across the cell culturing surface. In future work, we should investigate the effect of vibration amplitude on the cell collecting rate and improve the uniformity of the vibration amplitude over the cell culturing surface.

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