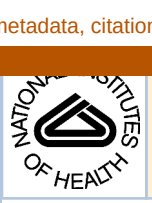



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# Microsphere-Based Scaffolds for Cartilage Tissue Engineering: Using Sub-critical $CO_2$ as a Sintering Agent[ξ]

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## Abstract

Shape-specific, macroporous tissue engineering scaffolds were fabricated and homogeneously seeded with cells in a single step. This method brings together $CO_2$ polymer processing and microparticle-based scaffolds in a manner that allows each to solve the key limitation of the other. Specifically, microparticle-based scaffolds have suffered from the limitation that conventional microsphere sintering methods (e.g., heat, solvents) are not cytocompatible, yet we have shown that cell viability was sustained with sub-critical (i.e., gaseous) $CO_2$ sintering of microspheres in the presence of cells at near-ambient temperatures. On the other hand, the fused microspheres provided the pore interconnectivity that has eluded supercritical $CO_2$ foaming approaches. Here, fused poly (lactide-co-glycolide) microsphere scaffolds were seeded with human umbilical cord mesenchymal stromal cells to demonstrate the feasibility of utilizing these matrices for cartilage regeneration. We also demonstrated that the approach may be modified to produce thin cell-loaded patches as a promising alternative for skin tissue engineering applications.

### Keywords

Microspheres; Sub-critical $CO_2$; Sintering; Cell-loaded implants; Cartilage tissue engineering

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## Introduction

Microsphere-based tissue engineering scaffold designs have attracted significant attention in recent years [1–7], as the microspheres as building blocks offer several benefits, including ease of fabrication, control over morphology and physicochemical characteristics, and versatility of controlling the release kinetics of encapsulated factors [8]. The properties of a scaffold, in turn, can be tailored by altering the microsphere design and fabrication method, for example to create gradient-based scaffolds [7,9,10]. Similarly, macromechanical properties and degradability can be altered with the selection of the raw material. The polymer also offers flexibility in the degradation kinetics, modulated by altering one or more of the factors, such as the molecular weight, co-polymer ratio, tacticity, crystallinity, etc. [11,12]. To date, the methods used to produce microsphere-based scaffolds have utilized heat-sintering [3,13], a solvent vapor treatment (dichloromethane) [14,15], a solvent/non-solvent sintering method (acetone and ethanol treatment) [16,17], or a non-solvent sintering technique (ethanol treatment) [7], all of which involve exposure to elevated temperatures or organic solvents that may be a potential limitation for their pharmaceutical or medical applications [18].

In lieu of conventional temperature-regulated or organic solvent-assisted scaffold fabrication, supercritical fluid (SCF)-technology has offered an alternative method of melt processing of the polymers [19]. Specifically, supercritical carbon dioxide ($CO_2$) has been widely used as it is inexpensive, non-toxic, non-flammable, recoverable and reusable [18]. Exploiting the ability of supercritical $CO_2$ ($T_c$ = 304.1 K, $P_c$ = 73.8 bar) to dissolve/plasticize many polymeric materials, some polymeric scaffold fabrication techniques have been developed including gas foaming and emulsion templating (see reviews by Davies *et al.* [19] and Barry *et al.* [20]), which allow incorporation of bioactive factors and/or cells during the scaffold fabrication [21,22]. Near-critical or liquid $CO_2$ exposure (pressure ~ 55–60 bar) has been used to produce regular or intricate-shaped scaffolds using gas foaming or particulate consolidation [23–25]. However, one inherent limitation of gas foaming-based techniques is the closed-cell structure and lack of pore interconnectedness, and alternative modified techniques such as gas foaming/particulate are usually time-consuming and present challenges in incorporating bioactive factors [19,26].

For microsphere-based scaffolds, microsphere size is one of the major determinants of polymer degradation rate, governing the release kinetics of loaded molecules and providing the control over pore-sizes and macro-porosity [7]. Utilizing our ability to create highly monodisperse microspheres [27] and capitalizing on the plasticizing ability of $CO_2$, a novel microsphere-based scaffold fabrication technique is presented here using poly(D,L-lactide-co-glycolide) (PLG) microspheres, which also allows the production of shape-specific scaffolds. Using chondrocytes and human umbilical cord mesenchymal stromal cells (HUCMSCs) [28–31], preliminary evaluations of the scaffolds for cartilage tissue engineering applications were performed. Perhaps most importantly, the $CO_2$ sintering technique is amenable to produce cell-containing, shape-specific matrices (patches and scaffolds) under relatively mild conditions via a single-step sintering of microspheres in the presence of cells, with high cell viability.

## Materials and methods

### $CO_2$ sintering of shape-specific and bimodal scaffolds

Uniform PLG (50:50 lactic acid:glycolic acid; acid end group, Mw ~40,000–45,000 Da of intrinsic viscosity (i.v.) 0.33 dL/g (Lactel, Pelham, AL) and of i.v. 0.37 dL/g (Lakeshore Biomaterials, Birmingham, AL)) microspheres were fabricated using technology from our previous reports [7,27]. The nominal particle sizes were: 120 μm, 140 μm (both with an i.v. of 0.37 dL/g), and 5 μm, 100 μm, 175 μm, 240 μm and 500 μm (i.v. = 0.33 dL/g). The size distribution of microspheres was determined using a Coulter Multisizer 3 (Beckman Coulter

Inc., Fullerton, CA). Particles of different average diameters were separately loaded into cylindrical molds, and exposed to $CO_2$ at sub-critical levels, commonly ~ 15 bar (220 psig) at 25°C for 1 h followed by depressurization at ~0.14–0.21 bar/s, unless otherwise specified. $CO_2$ exposure was accomplished with a high-pressure vessel, consisting of a stainless steel body with view windows rated to 400 bar of pressure.

Scaffolds containing a bimodal distribution of particles were prepared using a mixture of particles of two different sizes (5 μm and 140 μm). Preparation of shape-specific scaffolds was carried out in a similar manner in rubber molds cut into specific designs.

## Morphological assessment

For morphological assessment, freeze-dried scaffolds were sputter coated with gold and observed using a Leo 1550 field emission scanning electron microscope at an accelerating voltage of 5 kV. Mechanical characterization of the scaffolds (1 to 4 mm height, diameter ~6 mm) was performed under uniaxial, unconfined compression (Instron Model 5848, Canton, MA; 50 N load cell). Samples were tare-loaded (~10 kPa), then compressed at a strain rate of 0.5 mm/min under phosphate buffered saline (0.138 M sodium chloride, 0.0027 M potassium chloride) at 37°C [7]. Moduli of elasticity were obtained from the initial linear regions of the stress-strain curves [7,32].

## Cell harvest and seeding

Chondrocytes were harvested from hog ankles and mandibular condyles (Duroc breed, 6 months old, female) as described previously [28]. Frozen HUCMSCs (P1) for 3 week cell culture studies were generously donated by Dr. Mark Weiss's group at Kansas State University (The Kansas State University IRB approval no. 3966) [31]. All cells were plated for expansion in monolayer and incubated at 37°C in 5% $CO_2$, with media changed every 2–3 days. The culture medium for HUCMSCs was composed of Dulbecco's Modified Eagle medium (DMEM; low glucose), 1% penicillin–streptomycin (both from Invitrogen Life Technologies, Carlsbad, CA) and 10% fetal bovine serum (FBS; Gemini, West Sacramento, CA). The culture medium for chondrocytes consisted of DMEM (high glucose), 1% penicillin–streptomycin–fungizone, 1% non-essential amino acids (NEAA) (all from Invitrogen Life Technologies), 10% FBS and 25 μg/mL L-ascorbic acid (Sigma, St. Louis, MO). Chondrocytes from the porcine mandibular condyle (P3) and ankle (P2) were mixed before being seeded onto one set of scaffolds, whereas HUCMSCs (P4) were seeded onto another set of scaffolds.

## Measurement of cell performance on scaffolds

Cylindrical scaffolds (6 mm diameter, ~2 mm height) were produced using microspheres of 175 μm diameter at $CO_2$ sintering conditions of ~13 bar (190 psig) pressure and 1 hour exposure followed by depressurization at ~0.14–0.21 bar/s. Cells were then seeded on scaffolds (sterilized using ethylene oxide) at a density of approximately $20 \times 10^6$ cells per mL of scaffold using the orbital shaker method as described previously [33], and cultured for 3 weeks with half of the media refreshed every other day. During the cell culture, the culture medium for HUCMSC-seeded scaffolds was replaced with a chondrogenic medium [31]. At week 3, scaffolds were analyzed for matrix production using histology (Safranin-O staining for GAG production), immunohistochemistry (for collagen types I and II), and biochemical assays (picogreen, hydroxyproline, and dimethylmethylene blue (DMMB) assays for determining the cell number, collagen content and GAG content, respectively), as described previously [31]. Cell viability was evaluated with a LIVE/DEAD assay (2 mM calcein AM, 4 mM ethidium homodimer-1; Molecular Probes Carlsbad, CA) with fluorescence microscopy (Olympus/Intelligent Innovations Spinning Disk Confocal Microscope) [7].

### Evaluation of cell viability following single-step scaffold fabrication

To assess cell survival during sub-critical $CO_2$ sintering, HUCMSCs were harvested from one human umbilical cord obtained from the University of Kansas Medical Center (KU Medical Center IRB approval no. 10951, KU-Lawrence IRB approval no. 15402) as described earlier [31]. To prepare cell-loaded constructs, cell pellets of HUCMSCs (P4; $1 \times 10^6$ cells) were mechanically mixed with ethylene oxide-sterilized microspheres (diameter: 120 μm, ~200 mg) using a sterile spatula. The cell-particle mixture was loaded into cylindrical molds and exposed to $CO_2$ at subcritical conditions (30 bar, 4 min, 25°C, depressurization rate ~3psi/s). A modified processing was performed for another set of particles (size: 120 μm, ~100 mg), where the particles were suspended in 100 μL of medium containing HUCMSCs (P4; $2 \times 10^6$ cells), then exposed to $CO_2$ at subcritical conditions (30 bar, 2 min, 25°C, depressurization rate ~3psi/s). The prepared constructs were assessed for HUCMSC viability using the LIVE/DEAD staining as described above.

### Statistical analysis

The effects of microsphere size on the mechanical properties of the scaffolds were statistically analyzed using a seven-level single factor analysis of variance (ANOVA) followed by Tukey's Honestly Significant Difference *post hoc* test (n = 5, except for scaffolds prepared with 240 μm and bimodal spheres (n =4), and 140 μm (n = 6)).

## Results

Relatively monodisperse microspheres having uniform nominal diameters were created using a previously reported method [27]. These microspheres demonstrated a solid interior morphology [7]. Monodispersity was verified with all microsphere diameters: 120 μm, 140 μm (i.v. = 0.37 dL/g), and 5 μm, 100 μm, 175 μm, 240 μm and 500 μm (i.v. = 0.33 dL/g) (Fig. 1A). A variety of shape-specific scaffolds were also constructed using 140 μm microspheres (Fig. 1B). Morphological assessment of the scaffolds using scanning electron microscopy revealed that the microsphere matrices were porous, where the microspheres largely retained their shape (Fig. 2). Under the typical $CO_2$ sintering conditions employed, the extent of sintering of the microspheres was found, in general, to be a factor of the microsphere size (compare Fig. 2(A and B) with (C and D), respectively). Also, the PLG microspheres of lower i.v. (i.e., 0.33 dL/g) displayed a distortion from the spherical morphology and a higher degree of sintering (compare Fig. 2(A and C) with (B and D), respectively). Both the size of the microsphere and the intrinsic viscosity of the polymer were found to affect the pore sizes. As can be observed in Fig. 2, the pore sizes for the scaffolds prepared with PLG microspheres of lower i.v. had anisotropic pores with closed pores at several places. Roughly, the average pore-sizes were around 70 μm (Fig. 2A and 2B), 50 μm (Fig. 2C) and 40 μm (Fig. 2D). The different average pore sizes were reported here based on the SEM image analysis of the scaffolds using the annotation feature of the user interface program (Leo 1550), which is only an estimate. Micrographs of a single microsphere (140 μm) revealed the modifications in the surface of the microspheres following the $CO_2$ sintering, including the microsphere connection sites (Fig. 2E).

The average moduli of the scaffolds, determined from the stress-strain plots using the end of the initial linear regions before the onset of non-linear region (rationale explained later), ranged from 71 to 196 kPa (Fig. 3), which may be suitable for cartilage tissue engineering applications. A higher intrinsic viscosity of the polymer, a decreased average diameter of the microspheres, or inclusion of smaller-sized microspheres in the interstitial spaces, all resulted in an improvement in the average mechanical moduli of the scaffolds. The differences between the average moduli of all the groups, however, were not found to be statistically significant.

Cell culture studies were performed to determine the suitability of these scaffolds for tissue engineering. Porcine chondrocytes, dynamically seeded and cultured on the scaffolds, were assessed for their viability. The majority of the cell population was identified as viable after 3 weeks in culture (Fig. 4-I). Immunohistochemistry revealed positive staining for collagen types I and II following the 3 week culture (Fig. 4-II). In addition, Safranin-O staining revealed signs of glycosaminoglycan (GAG) formation for both the groups (Fig. 4-II). Biochemical analysis also revealed positive indications of cartilage-like matrix formation, where the presence of GAGs and collagen were detected (Table 1). In addition, cell loaded matrices were fabricated via a one-step $CO_2$ sintering of microspheres with the HUCMSCs. Under a brief $CO_2$ exposure time and at relatively low $CO_2$ pressures, homogeneously-seeded three-dimensional constructs or thin patches were prepared in the absence or presence of culture medium, respectively (Fig. 5). Viability assessment of the cell-loaded thin patch and the scaffolds revealed that virtually the entire cell population survived the sintering process (Fig. 5B and C).

## Discussion

The sub-critical $CO_2$ sintering method to manufacture microsphere-based scaffolds is a modification of the gas foaming technique. In the past, plasticization of PLG in pressurized $CO_2$ has been applied to create foamed scaffolds, where saturation of the polymer with $CO_2$ was performed at sub-critical pressures (~55 bar) with equilibration periods of greater than 24 h, and a rapid depressurization led to the nucleation of the gas (forming pores in the material) and restoration of the glass transition temperature [23,26,34]. To prepare microsphere-based matrices in the current study, the equilibration of $CO_2$ in the polymer was restricted by decreasing the pressure and the duration of $CO_2$ exposure, leading to a comparatively reduced plasticized state or a relatively less swollen state of the PLG. While this allowed the microspheres to primarily retain their shape, the swelling of the microsphere surfaces and subsequent adhesion and/or reptation led to the sintering of the adjoining microspheres, yielding a porous matrix (Fig. 2). The conditions of $CO_2$ exposure are likely a prime factor responsible for promoting the mutual-penetration and increasing the chain mobility at the interfaces of adjoining microspheres [35]. Based on preliminary investigations, the pressure (15 bar) and duration of $CO_2$ exposure (1 h) were selected to allow sintering of all of the microspheres (with different sizes and i.v. of PLG). Usually, microspheres with smaller sizes may require milder conditions (less pressure or shorter exposure) to achieve optimal sintering. As shown earlier, a similar consolidation technique applied to attach PLG fiber aggregates (700–1400 μm) required only a 15 s duration, however with liquid $CO_2$ at 55 bar pressure [25]. In addition, the rate of depressurization was an important factor that governed the basic morphology of the scaffolds in the current study. A moderate rate of depressurization (0.14–0.21 bar/s) was found to be optimal for the production of sintered matrices. For typical $CO_2$ sintering conditions, instantaneous depressurization (i.e., in less than 5 s; for 64 μm diameter microspheres, i.v. = 0.33 dL/g) or depressurization at very slow rates (i.e., < 0.07 bar/s; for 240 μm diameter microspheres, i.v. = 0.33 dL/g) led to foaming of the prepared scaffolds, depending on the microsphere size and i.v. of the polymer.

In general, the morphology of the scaffolds produced in the present work closely resembled the morphology of the microsphere-based scaffolds produced using an ethanol sintering method, reported earlier [7]. The ethanol-sintered scaffolds had an approximate porosity of ~41% and were isotropic (degree of anisotropy 1.06) with an interconnected pore structure, which may provide an estimate for the scaffolds produced using the $CO_2$ sintering method. The microsphere morphology, closely resembling the appearance of a microsphere reported earlier in the ethanol sintering method [7], showed the presence of a surface film of PLG containing ripples, indicating the surface swelling of PLG (Fig. 2). To improve the inter-microsphere connection that could improve the mechanical characteristics of the scaffolds, scaffolds were prepared using two different groups of microspheres (140 μm and 5 μm) (mixed

together in a ratio of 1:8 by weight, respectively). Additional connecting bridges between the large microspheres were formed, however, at the loss of overall scaffold porosity, with reduced pore-sizes (Fig. 2F).

Mechanical characterization of the scaffolds was performed by unconfined compression under simulated physiological conditions. The hypothesized mechanism of compression for microsphere-based matrices is somewhat analogous to the compression of closed-foam cellular solids [32]. Following an initial linear region, a non-linear pore collapse region follows [7]. The moduli of elasticity were determined from the stress-strain plots using the end of the initial linear regions before the onset of non-linear region (extending to ~ 40% strain, in general), which indicate the scaffold elasticity [7]. The stiffness of the scaffolds revealed a somewhat inverse relationship between average microsphere size and average mechanical modulus. Also, a higher intrinsic viscosity of the polymer also seemed to improve the mechanical characteristics, probably because of a spherical morphology and more ordered packing of the microspheres (as mentioned before, see Fig. 2). In addition, inclusion of smaller interstitial spheres in the pores led to an increase in the average mechanical modulus of the scaffolds.

Porcine chondrocyte culture demonstrated the feasibility of using the scaffolds prepared using our sub-critical $CO_2$ sintering method for cartilage tissue engineering application. However, biochemical analysis revealed that the cell number per construct considerably dropped (Table 1), and the majority of the cells could not attach to the scaffolds. A possible reason could be the cell seeding method, where cell infiltration into the scaffolds was probably affected by seeding them dynamically [7]. To address this issue and to allow for homogeneous seeding of the constructs, cell loaded matrices were fabricated via a one-step $CO_2$ sintering of microspheres with the HUCMSCs. The conditions of sintering were altered to minimize the time of exposure (4 min or less, excluding the depressurization time), while keeping the $CO_2$ pressure to a relatively low value (30 bar). Interestingly, when performed in the presence of the culture medium, the sintering process resulted in a thin patch formation, where only a few microsphere layers at the top of the mold were sintered together (Fig. 5). In contrast, in the absence of the medium, a mixture of cells with the microspheres yielded completely sintered matrices. The difference between the thin patch formation (with culture medium) and full 3D scaffold formation (absence of medium) can be attributed to the thermodynamic limitation of $CO_2$ solubility in the liquid phase (Henry's Law). Qualitative viability assessment of the cell-loaded thin patch and the scaffolds indicated close to 100% cell survival following the sintering process (Fig. 5B and C). Further quantitative assessment will, however, be required in the future to compare with these observations. The pioneering work by Ginty *et al.* [21], where cell survival in a brief exposure to supercritical $CO_2$ was demonstrated, and less than 5 min of overall $CO_2$ exposure was shown to be primarily non-malignant for a variety of cells, formed the basis of selecting the exposure time range. Although $CO_2$ at high pressures for long durations may not be cytocompatible due to known sterilization efficacy of supercritical $CO_2$ achieved by lowering the cytoplasmic pH from the formation of carbonic acid and the shear forces of intercellular bubble formation upon depressurization, we have demonstrated that the milder conditions with milder gaseous $CO_2$ conditions are highly conducive to cell viability. Based on the size of the microspheres, the type of PLG, and the type of cells under consideration, various sub-critical $CO_2$ sintering conditions may exist (i.e., a number of combinations of sub-critical pressures and exposure times), which may allow the formation of cell-loaded matrices without affecting the cell viability.

In the present work, *in vitro* evaluations were performed only on a selected scaffold type, where the goal was to demonstrate the feasibility of utilizing these matrices for cartilage tissue engineering. A preliminary study demonstrated that the scaffolds made from the microspheres of diameter 175 μm allowed cellular infiltration, which formed the criterion for selection of these scaffolds for the cell culture studies. However, microsphere size is a factor that directly

affects the pore sizes of the resulting matrices, thereby may directly affect the cellular infiltration and cell-to-cell interaction [7]. Therefore, future efforts will be needed to characterize the biological performance of the scaffolds as a function of microsphere size. Moreover, studies will be warranted to evaluate both a broad spectrum of process parameter combinations (including, $CO_2$ pressure and exposure time) and evaluation of matrix synthesis in long-term studies. Furthermore, the approach to *a single-step cell-biomaterial construct fabrication* in the presence or absence of medium will require further investigations *in vitro* and quantitative evaluation of cell viability and matrix synthesis to identify the long-term performance of cell-loaded constructs prepared in this manner.

## Conclusions

Gaseous $CO_2$ sintering was found to be a straightforward method to fabricate cell-seeded, microsphere-based, shape-specific constructs in a single step. These constructs of course retain the numerous advantages of microsphere-based scaffolds such as spatiotemporal control for creating 3D signal and stiffness gradients for interfacial tissue engineering within a single scaffold. Compared to the other methods of microsphere-based scaffold fabrication, which utilize heat, solvent and/or anti-solvent–induced plasticization [3,7,13–17], the $CO_2$ sintering method may be a more benign process. The resulting scaffolds were porous, exhibited moduli similar to the native cartilaginous tissues, and displayed support for chondrogenesis and cartilage-like tissue growth. The process of sub-critical $CO_2$ sintering is also amenable to producing cell-containing matrices under relatively mild conditions. The ability to create cell-loaded scaffolds and patches may have important implications for cartilage and skin tissue engineering, respectively, where growth factor-encapsulated microspheres can be used to design cell-loaded controlled release vehicles in a single-step.

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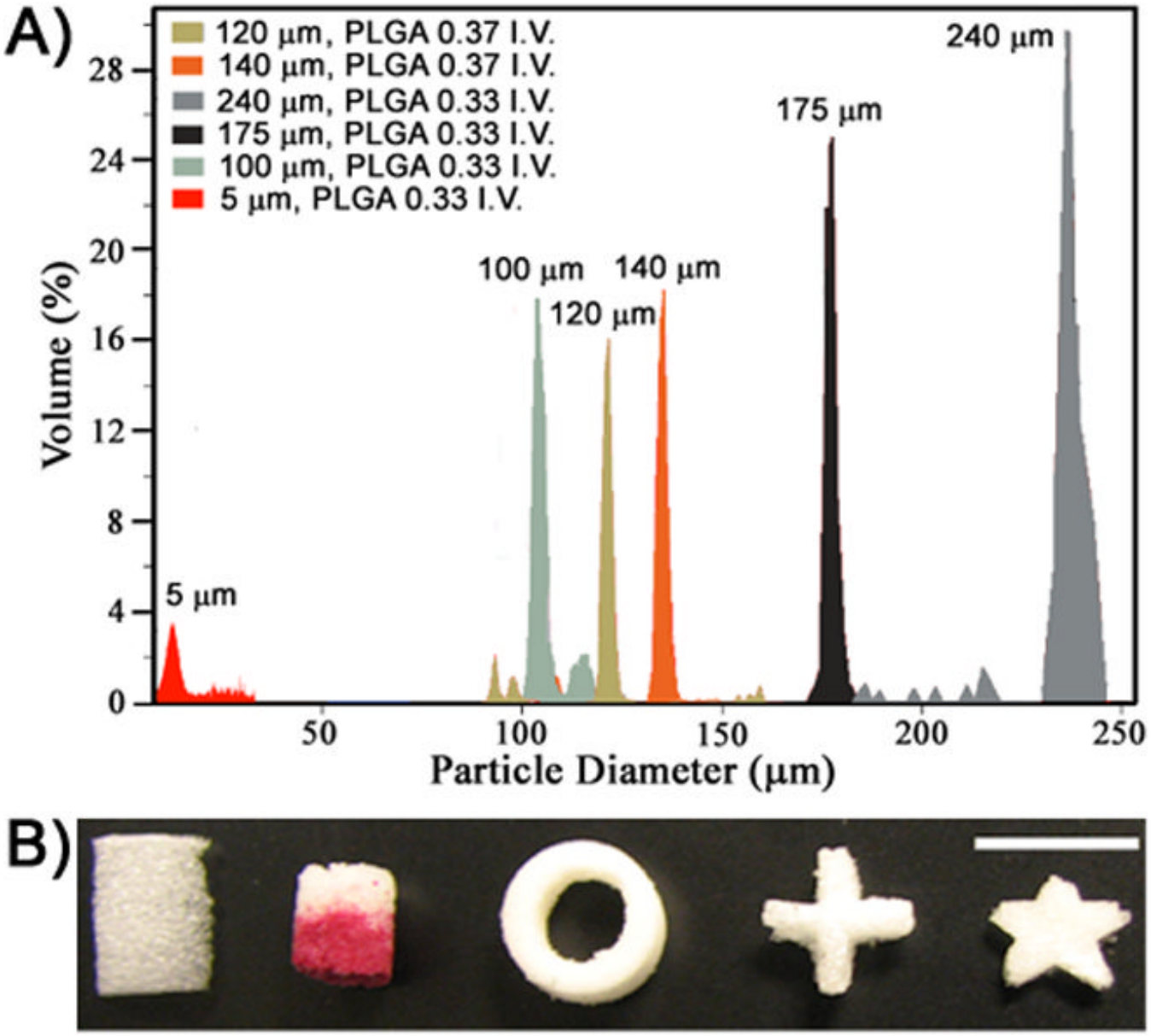


**Figure 1.**

(A) Coulter multisizer size distribution plot of PLG microspheres of different nominal sizes used in these studies, displaying the monodispersity of the microspheres with discrete peaks (peaks with % volume less than 0.5 have been omitted for the sake of clarity). (B) An image of various shape-specific scaffolds that were produced with PLG microspheres (140 µm) using $CO_2$ at sub-critical conditions (15 bar for 1 h at 25°C followed by depressurization at ~0.14–0.21 bar/s) utilizing rubber molds of different shapes. From left to right: cylinder, bilayered cylinder, tube, plus-sign, and star. Scale bar: 1 mm.

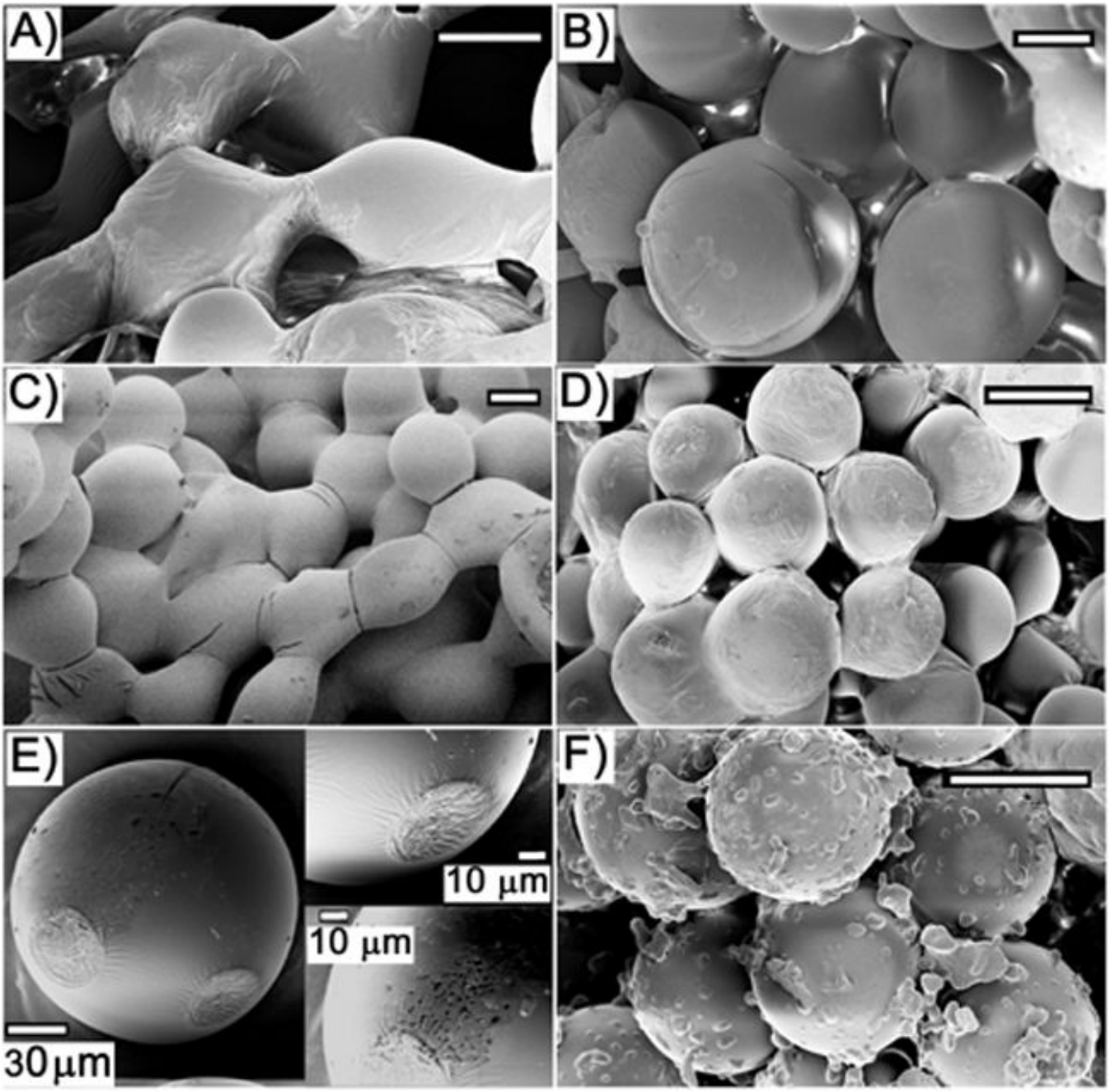


**Figure 2.**

Characteristic scanning electron micrographs of scaffolds fabricated using different types of PLG microspheres at the typical processing conditions for sintering ($CO_2$ exposure at 15 bar for 1 h at 25°C followed by depressurization at ~0.14–0.21 bar/s). Sizes of the microspheres used were 240 μm (A, B), 175 μm (C), 140 μm (D, E), and 140 μm together with 5 μm (F). The morphology of a microsphere following the $CO_2$ sintering (E) is also displayed, where enlarged images of the microsphere connection site (top panel) and sub-micron level surface modifications (bottom panel) are shown. The microspheres were made using PLG (with acid-end group chemistry) of either 0.33 (5, 175, 240 μm) or 0.37 (140 μm) dL/g intrinsic viscosity (i.v.) (see Fig. 1). Scale bar: 100 μm unless labeled otherwise.

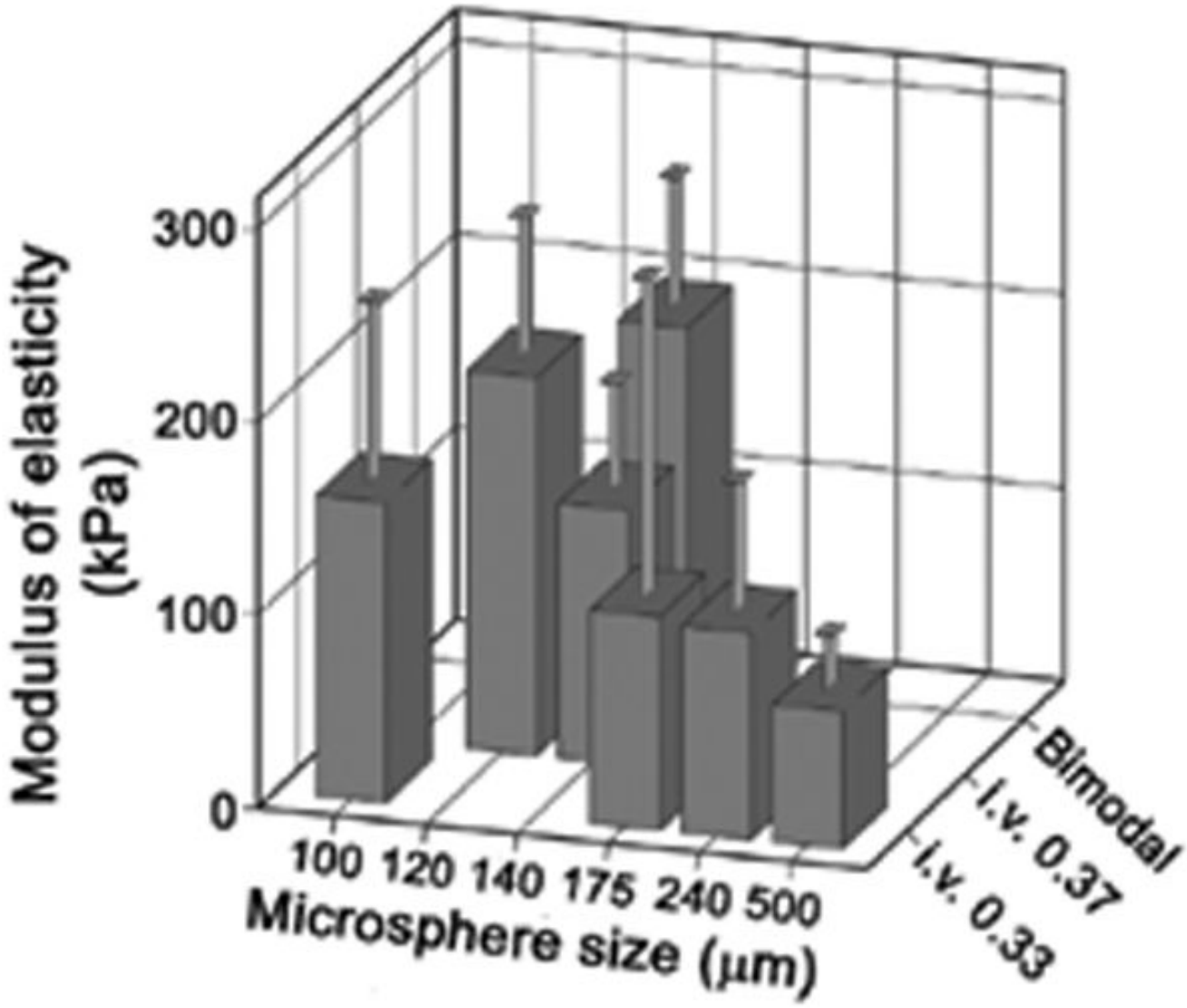


**Figure 3.**
Modulus of elasticity of the scaffolds prepared using different microsphere sizes (corresponding to Fig. 1). The differences in the moduli were not statistically significant ($p > 0.05$).

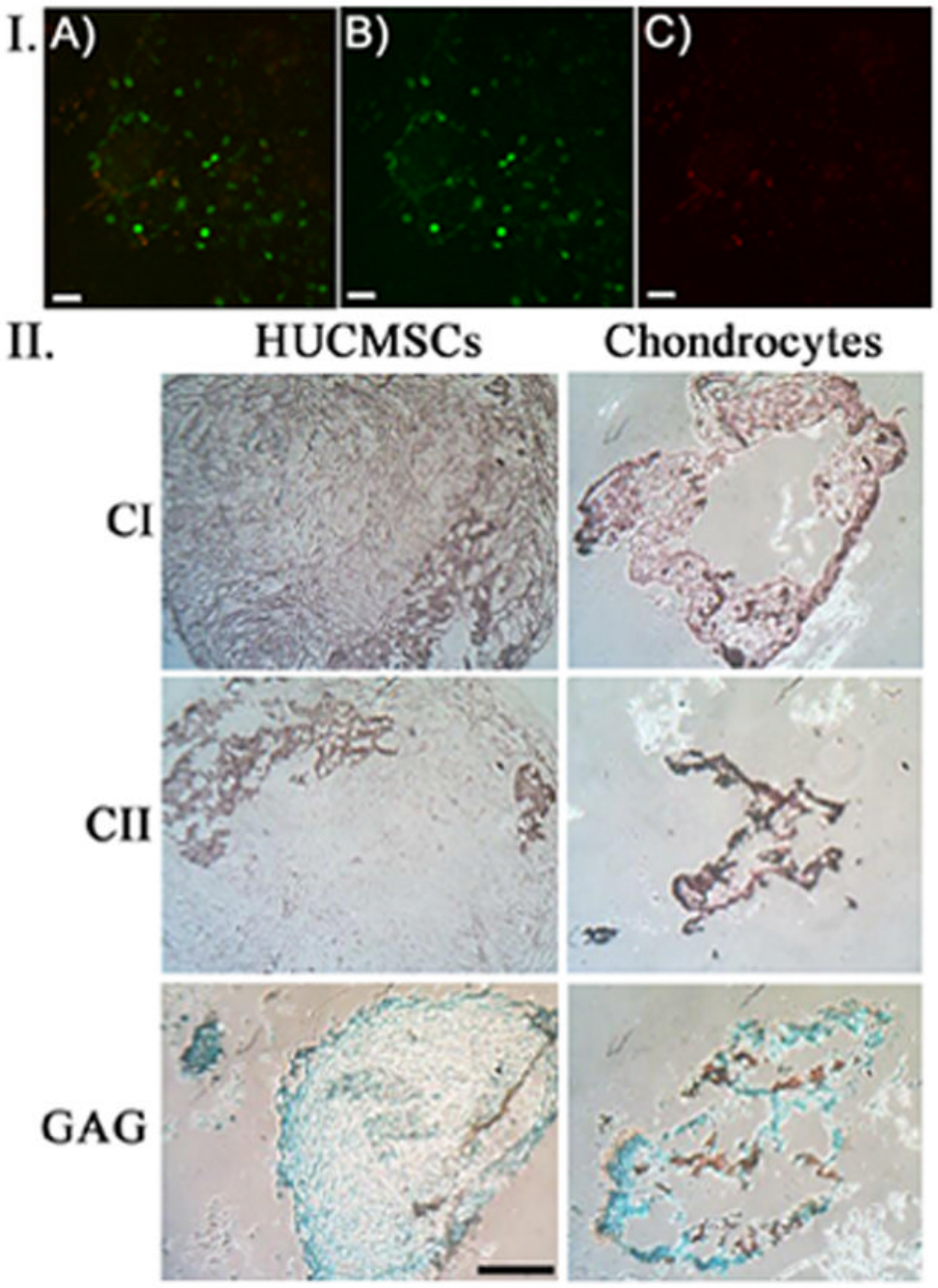


**Figure 4.**

I. Fluorescence micrographs of Live/Dead dye-stained porcine chondrocytes seeded on scaffolds (175 μm) following a 3 wk cell culture period – A) live (green) and dead (red) cells. Panels B and C show the split of green and red, where all the live cells (B) or dead cells (C) can be seen separately, respectively. There is a mild overlap between the panels B and C, which reveals some cells that are yellow (possibly cells that are dying). Scale bar: 100 μm. II. Immunohistochemistry for collagen types I and II (purple indicates positive stain) and Safranin-O staining for GAGs (orange indicates positive stain) at week 3 (n = 2). HUCMSCs = human umbilical cord mesenchymal stromal cells, CI = collagen type I, CII = collagen type II, and GAG = glycosaminoglycan. Scale bar: 100 μm.

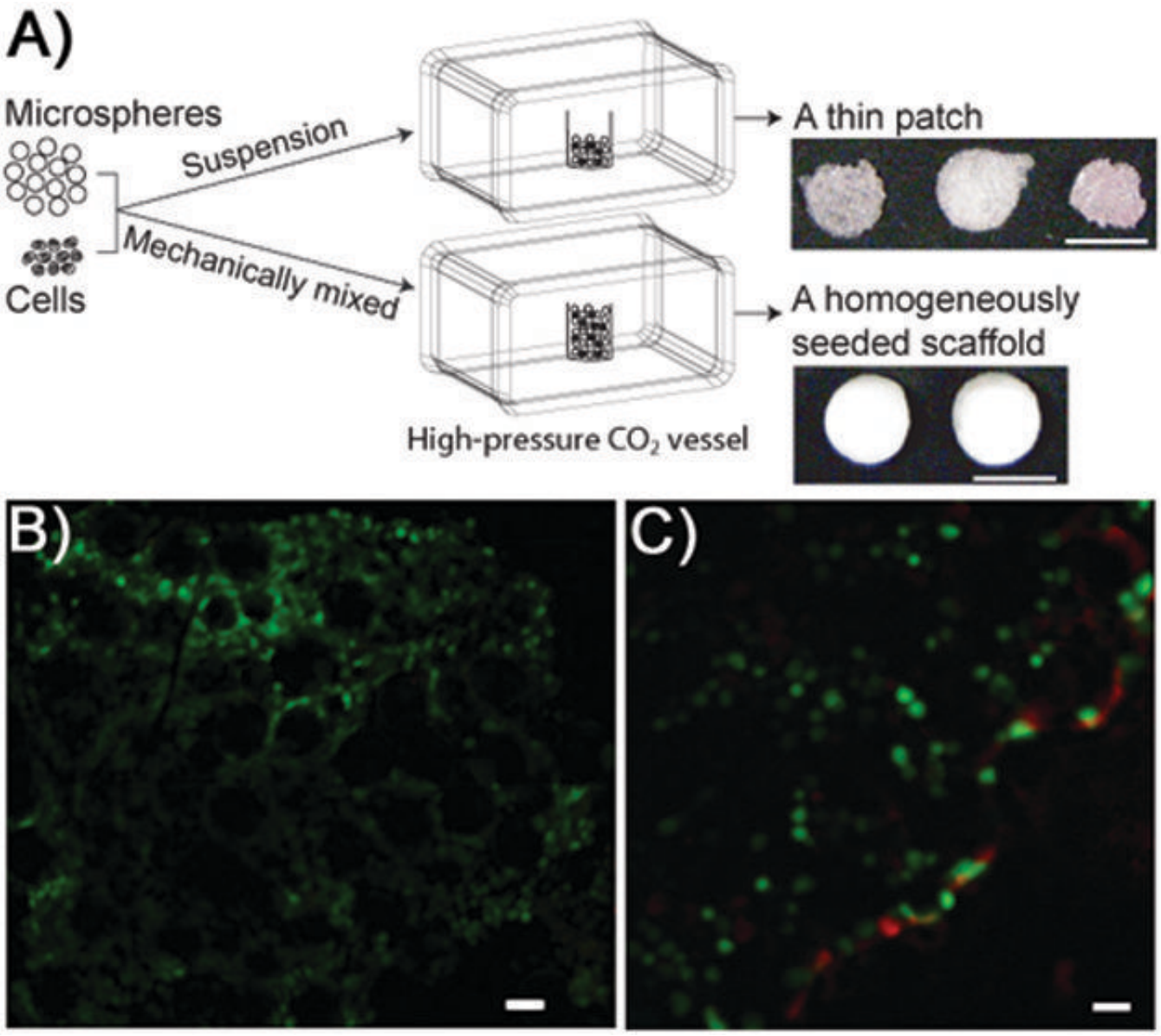


**Figure 5.**

(A) A schematic of producing a microsphere-based cell-loaded scaffold or thin patch is shown. The process of combining the cells and microparticles in a liquid medium results in a melded thin patch (top), whereas mechanically mixing a loose cell pellet in a minimal liquid volume with the microparticles results in a homogeneously seeded scaffold (bottom). Scale bar: 6 mm. (B, C) Fluorescence micrographs of Live/Dead dye-stained HUCMSCs display cell survival during $CO_2$ sintering of microspheres (120 μm) at sub-critical conditions. Processing conditions (pressure, duration of exposure, depressurization rate, presence/absence of culture medium) for the production of the thin patch (B) and the macroscopic scaffold (C) were (30 bar, 2 min, ~0.21 bar/s, medium present) and (30 bar, 4 min, ~0.21 bar/s, medium absent), respectively. Green indicates live cells and red indicates dead cells. Note the dark circular areas, corresponding to the locations of the microspheres. Scale bar: 100 μm.

**Table 1**

Biochemical assay results following 3 wk cell culture[a]

| Scaffold Group | Number of cells | GAG content (μg) | Hydroxyproline content (μg) |
|---|---|---|---|
| Chondrocytes | $5.8 \pm 1.0 \times 10^4$ | 12.8 ± 7.1 | 1.8 ± 0.8 |
| HUCMSCs | $5.9 \pm 1.0 \times 10^4$ | 2.8 ± 1.0 | 1.8 ± 0.4 |

[a]Mean ± S.D.; n = 4, except for cell number for the chondrocyte-seeded group with n = 3.